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نموذج رقم (١٨)
اقرار والتزام بالمعايير الأخلاقية والأمانة العلمية
وقوانين الجامعة الأردنية وأنظمتها وتعليماتها
لطلبة الماجستير

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عنوان الرسالة:
Vitamin D Receptor Gene Polymorphism among Jordanian Patients with Type 2 Diabetes Mellitus

اعلن بأنني قد التزمت بقوانين الجامعة الأردنية وأنظمتها وتعليماتها وقراراتها السارية المفعول المتعلقة باعداد رسائل الماجستير عندما قمت شخصيا" باعداد رسالتي وذلك بما ينسجم مع الأمانة العلمية وكافة المعايير الأخلاقية المتعارف عليها في كتابة الرسائل العلمية. كما أنني أعلن بأن رسالتي هذه غير منقولة أو مستلة من رسائل أو كتب أو أبحاث أو أي منشورات علمية تم نشرها أو تخزينها في أي وسيلة اعلامية، وتأسيسا" على ما تقدم فانني أتحمل المسؤولية بأنواعها كافة فيما لو تبين غير ذلك بما فيه حق مجلس العمداء في الجامعة الأردنية بالغاء قرار منحي الدرجة العلمية التي حصلت عليها وسحب شهادة التخرج مني بعد صدورها دون أن يكون لي أي حق في التظلم أو الاعتراض أو الطعن بأي صورة كانت في القرار الصادر عن مجلس العمداء بهذا الصدد.

توقيع الطالب: داليا التاريخ: ١٧ / ٠٨ / ٢٠١١

VITAMIN D RECEPTOR GENE POLYMORPHISM AMONG JORDANIAN PATIENTS WITH TYPE 2 DIABETES MELLITUS

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This Thesis was submitted in Partial Fulfillment of the requirements for the Master's Degree in Medical Laboratory Science.

Faculty of Graduates Studies
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COMMITTEE DECISION

This Thesis/Dissertation (VITAMIN D RECEPTOR GENE POLYMORPHISM AMONG JORDANIAN PATIENTS WITH TYPE 2 DIABETES MELLITUS) was Successfully Defended and Approved on August, 11, 2011.

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DEDICATION

This thesis is dedicated to my parents, Dr. Khalil Hanash and Hana Karzoun, who taught me the value of education and introduced me to the joy of reading from birth. I am deeply indebted to them for their sacrifices, never-ending moral support and prayers which were catalysts to my achievements. To my mother, my sincere gratitude for her love, support, and encouragement throughout my life. Very special thanks for providing a 'writing space' and for nurturing me through the months of writing. I would like to give my heartfelt appreciation to my father who has been a source of continuous encouragement and inspiration to me throughout my life. He is the most wonderful father a girl could ever have, and I feel so special to be his daughter.

I would like to convey my gratitude to my sister, Dareen, and to my brother, Jawad, for their assistance, support, and understanding, to my brother, Mohammed, who make our home the warmest place to stay through his caring ways, and to my cousin Rami for his help.

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This is only the beginning of my journey.

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LIST OF ABBREVIATIONS

ADA: American diabetes ssociation

ANP: Arterial natriuretic peptide

BMI: Body mass index

Bp: Base pair

BSA: Bovine serum albumin

CAD: Coronary artery disease

CI: Confidence interval

DBD: DNA binding domain

DBP: Vitamin D–binding protein

DNA: Deoxyribonucleic acid

DRIP205: Vitamin D receptor-interacting proteins 205

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme liked immunosorbent assay

ESRD: End stage renal disease

FBS: Fasting blood sugar

FPG: Fasting plasma glucose

HPLC: High-performance liquid chromatography

HTN : Hyperension

IGT: Impaired glucose tolerance

IFG: Impaired fasting glucose

IL: Interleukin

IRS-1: Insulin receptor substrat-1

JUH: Jordan university hospital

LC-MS/MS: Liquid chromatography tandem mass spectroscopy

NCDEG: National center of diabetes, endocrinology and genetics

OD: Optical density

OGTT: Oral glucose tolerance test

OR: Odds ratio

PCR: Polymerase chain reaction

PI3Kinase: Phosphatidylinositol 3-kiase

PTH: Parathyroid hormone

RBC: Red blood cell

RFLP: Restriction fragment length polymorphism

RIA: Radioimmunoassay

SARC: Steroid receptor coactivator

SD: Standard deviation

SNPs: Single nucleotide polymorphisms

Single nucleotide polymorphisms: is a DNA sequence variation occurring when a single nucleotide in the genome is altered. It may fall within coding sequences of genes, noncoding regions of genes, or in the intergenic regions. It usually doesn't cause overt debilitating disease. It has a frequency of 1 % or more in the population, if the frequency is lower than 1 %, the allele is regarded as mutation.

TBE: Tris-boric acid EDTA

T2DM: Type 2 diabetes mellitus

TFIIB: Transcription factor

TSH: Thyroid stimulating hormone

VDR: Vitamin D receptor

VDREs: Vitamin D-responsive elements

USA: United state of America

UVB: Ultra violet B

UV: Ultra violet

WBC: Wight blood cell

HbA1c: Hemoglobin A1c.

LIST OF APPENDICES

VITAMIN D RECEPTOR GENE POLYMORPHISM AMONG JORDANIAN PATIENTS WITH TYPE 2 DIABETES MELLITUS.

By
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Supervisor
Dr. Azmi M. Mahafzah, Prof.

ABSTRACT

Background: Vitamin D deficiency has been shown to play an important role in the pathogenesis of several diseases. The aim of this study was to determine the frequency of vitamin D receptor gene polymorphism (VDR) among Jordanian patients with type 2 diabetes mellitus, and to investigate the relationship between VDR gene polymorphism and disease variables.
Methods: The study included 200 patients with type 2 diabetes mellitus attending the Diabetes Clinics of the Jordan University Hospital (JUH) and the National Center for Diabetes, Endocrinology and Genetics (NCDEGs), and 200 nondiabetic nonobese persons who represented as a control group. All patients and controls have signed an informed consent form to participate in this study. Blood samples were collected from patients and controls and genomic DNA was extracted. Polymorphisms of the vitamin D receptor (VDR) gene were analyzed by DNA amplification with polymerase chain reaction (PCR) and endonuclease digestion with BsmI, TaqI, and ApaI enzymes. Clinical and laboratory data including weight, height, systolic blood pressure, diastolic blood pressure, age ,other diseases that patients had, fasting blood glucose, HbA1C, urea, creatinine, sodium, potassium, calcium and vitamin D levels were obtained from patients medical files.
Results: Overall patients had a higher vitamin D level than controls. No significant difference was observed in the FBS levels or HbA1c according to vitamin D levels. In terms of vitamin D levels, no significant difference was observed between diabetic patients in relation to hypertension, obesity and coronary artery disease. The distributions of genotype frequencies of BsmI, TaqI, and ApaI polymorphism did not differ between diabetics and controls, but the frequency of Bb genotype of BsmI was significantly lower in diabetics (p=0.013). No statistically significant difference was observed in male and female patients compared to male and female controls in terms of the BsmI, TaqI and ApaI genotype polymorphisms. No statistically significant difference was observed between BsmI, TaqI and ApaI genotypes in terms of systolic blood pressure. A significant difference was, however, observed between BsmI genotypes in terms of diastolic blood pressure, which was significantly higher in patients with heterozygous and homozygous variant genotypes (AG+GG) than in patients with homozygous wild genotype (AA) of this polymorphism. Higher diastolic blood pressure was also observed in the heterozygous and homozygous variant genotypes (GT+TT) than in the homozygous wild genotype (GG) of ApaI

polymorphism. No significant associations were observed between the BsmI, TaqI and ApaI genotype polymorphisms and FBS or HbA1c levels. No significant difference was observed between diabetic patients in relation to hypertension and coronary artery disease. Nonobese diabetic patients had higher prevalence of (AA) wild genotype of BsmI polymorphism than obese diabetic patients whereas obese or overweight diabetic patients had higher prevalence of (GG) homozygous variant genotype of the polymorphism than nonobese patients. Obese or overweight diabetic patients had higher prevalence of (CT) heterozygous variant genotype of TaqI polymorphism than nonobese patients.

Conclusion: Vitamin D levels among diabetic patients of this study are higher than those among controls. Vitamin D receptor polymorphisms seem to have no effect on the pathogenesis of type 2 diabetes mellitus or on the pathogenesis of coronary artery disease among diabetic patients. On the other hand, vitamin D receptor polymorphisms might play a role in the pathogenesis of hypertension and obesity among diabetic patients.

INTRODUCTION

Vitamin D is a fat-soluble molecule that can be obtained from the dietary sources of vegetable in the form of vitamin D2, (also known as ergocalciferol) or animal origin in the form of vitamin D3, (also known as cholecaliferol) (Pérez-López, 2009, Zhang *et al.*, 2010). Only a small amount (30%) of vitamin D need can be obtained from the diet, since few food types contain it naturally. As a result, overall vitamin D requirements are covered from the sunlight-induced photochemical conversion of 7-dehydrocholesterol (Palomer *et al.*, 2008, Mathieu *et al.*, 2005, Judd and Tangpricha, 2009). Vitamin D3 itself is biologically inert and requires two successive hydroxylations; one in the liver (on C25) and one in the kidney (on the alpha position of C1) to form its hormonally active metabolite, 1, 25 dihydroxyvitamin D3 (Palomer *et al.*, 2008, Mathieu *et al.*, 2005).

Vitamin D increases the gut uptake of ingested calcium and phosphorus and improves calcium reabsorption by the kidney, thus resulting in the elevation of both mineral elements in plasma. As a consequence, the major biological actions of this vitamin include maintenance of mineral homeostasis and regulation of bone remodeling (Chiu *et al*. 2001, Palomer *et al*. 2008, Mathieu *et al*., 2005, Deluca *et al*., 2001). Many other vitamin D targets have been reported, such as heart, stomach, liver, brain, skin, pancreatic islets (β cells), thyroid, parathyroid and adrenal glands and immune cells (Palomer *et al*., 2008). The 1, 25 dihydroxyvitamin D_3 molecule exerts its effect by activating vitamin D receptor (Palomer *et al*., 2008, Mathieu *et al., 2005*, Kato, 2000). The binding of 1, 25 dihydroxyvitamin D_3 to vitamin D receptor leads to the transcription of genes regulated by 1, 25 dihydroxyvitamin D_3. Vitamin D receptor (VDR) is a member of the steroid/thyroid hormone receptor family that functions as a transcriptional activator of many genes. Vitamin D

receptor usually binds as a heterodimer with the retinoic X receptor (RXR) (Chiu *et al*., 2001, Palomer *et al*., 2008, Mathieu *et al*., 2005). In fact, vitamin D receptor is expressed on a large number of tissues, including those involved in the regulation of glucose metabolism, such as muscle and pancreatic β cells. The vitamin D receptor gene, which is located on chromosome 12q13.1, consists of 14 exons, and shows extensive polymorphisms, including a mononucleotide $(A)_n$ repeat polymorphism in the 3′ untranslated region and four RFLPs: FokI within exon 2, BsmI and ApaI within successive introns between exon 7 and exon 9, and TaqI within exon 9 (Palomer *et al*. 2008, Mathieu *et al*. 2005).

Type 2 diabetes mellitus (T2DM), also known as non insulin dependent diabetes mellitus, is a group of metabolic diseases characterized by hyperglycemia as a result of an individual's resistance to insulin with an insulin secretory defect. This resistance results in relative, not an absolute, insulin deficiency (Bishope *et al*., 2010). There is evidence to suggest a role for vitamin D in insulin secretion and insulin sensitivity, which includes; the presence of vitamin D receptor and the vitamin D–dependent calcium-binding proteins (DBP) on pancreatic β-cells, the expression of 1-*α*-hydroxylase enzyme, which catalyzes the conversion of 25 hydroxyvitamin D to1, 25-dihydroxyvitamin D ($1,25(OH)_2D$) in pancreatic *β*-cells, the presence of vitamin D response element in the human insulin gene promoter and the presence of vitamin D receptor in skeletal muscles (Alvarez and Ashraf, 2010). In addition, 1, $25(OH)_2D$ directly activates transcription of the human insulin receptor gene, activates peroxisome proliferator activator receptor-*δ*, stimulates the expression of insulin receptor, and enhances insulin-mediated glucose transport in vitro (Alvarez and Ashraf, 2010).

Several studies have demonstrated a link between vitamin D receptor gene polymorphisms and type 2 diabetes, and these polymorphisms may influence insulin secretion and sensitivity. Although relatively few studies have been conducted, results have varied (Mathieu *et al*. 2005).

The objective of this study is to determine the frequency of VDR gene polymorphism among Jordanian patients with type 2 diabetes mellitus, and to investigate the relationship between VDR gene polymorphism and disease variables.

LITERATURE REVIEW

2. VITAMIN D

2.1 VITAMIN D HISTORY

The discovery of vitamin D was the result of scientists' efforts to find a cure for Rickets. Mellanby (1919) attributed the ability of cod liver oil to cure the rachitic condition in dogs as being another property of vitamin A. In 1922, McCollum destroyed the activity of vitamin A in cod liver oil by bubbling oxygen through the solution and heating it, but the ability to cure rickets remained in the preparation. McCollum and his co-workers correctly concluded that this represented a new vitamin. Because the water-soluble factors then discovered were termed vitamin B and the known anti scurvy factor was called vitamin C, they named the new factor vitamin D (Wolf, 2004, Rajakumar, 2003).

Huldshinsky *et al*., (1919) and Chick *et al*, (1923) independently demonstrated that rachitic children could be cured by exposure to sunlight or artificially produced ultraviolet light. The puzzle was ultimately solved when Steenbock and Black, (1924) discovered that irradiation not only of the skin of animals but also of the food they consumed imparted antirachitic activity to either animals or their food. Furthermore, Goldblatt and Soames (1923) showed that livers taken from irradiated rats could heal rickets in rats. The structure of vitamin D_2 was deduced by Askew *et al*. in 1931. Windaus *et al*. (1936) isolated and identified the compound 7-dehydrocholesterol, the irradiation product of which was named vitamin D_3, or Cholecalciferol. By 1967, the concept that vitamin D is converted to an active form had developed. By 1969, the circulating form of vitamin D had been isolated, chemically identified, and synthesized. This compound, 25-hydroxyvitamin D_3 ($25(OH)D_3$), is now monitored in serum to indicate vitamin D status of patients (DeLuca *et al*., 2004). The final active

hormone derived from vitamin D was isolated and identified in 1971, and its structure was deduced as 1, 25-dihydroxyvitaminD_3 (1, $25(OH)_2D_3$) and it was confirmed by synthesis (DeLuca *et al*., 2004).

2.2.VITAMIN D PHOTOSYNTHESIS AND METOBOLISM

Vitamin D is a seco-steroid hormone (Omdal *et al.,* 2002). The two major forms of Vitamin D are vitamin D_3 (cholecalciferol) and Vitamin D_2 (ergocalciferol) (Mathieu *et al*., 2005). Vitamin D_3 is the natural form of vitamin D produced in skin, and vitamin D_2 is derived from irradiation of ergosterol (Deluca, 2004). Few food types naturally contain vitamin D, including oily fish such as salmon, mackerel, and herring and oils from fish, including cod liver oil. In some countries, milk, some juice products, some bread, yogurt, and cheese are fortified with vitamin D (Thacher and Clarke, 2011, Holick, 2004). The major source of vitamin D is exposure to sunlight (Holick, 2004). During exposure to sunlight, the 7-dehydrocholesterol, the immediate precursor in the cholesterol biosynthetic pathway, in the epidermal and dermal cells absorbs ultraviolet B (UVB) irradiation with wave length of 290-315 nm (Holick, 2007). The absorption of this irradiation causes a break in the B-ring to form 9,10-seco-sterol, previtamin D_3. Previtamin D_3 is thermodynamically unstable, and it rearranges its double bonds and isomerizes to form the more thermodynamically stable vitamin D_3 structure (Holick, 2004). Once formed, vitamin D_3 is ejected into the extracellular space (Holick, 2004).

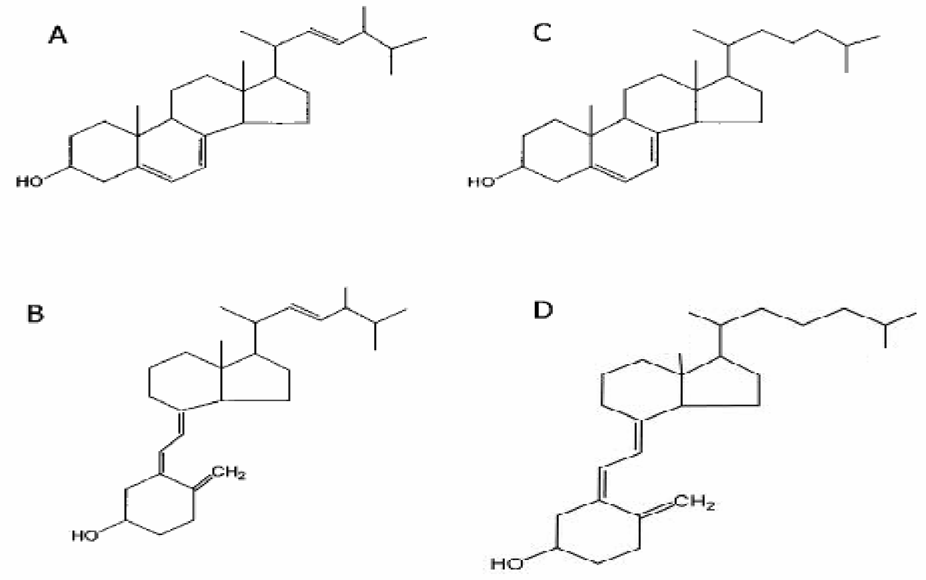


Figure 1: Structure of ergosterol (*A*), vitamin D-$_2$ (*B*), 7-dehydrocholesterol (*C*), and vitamin D-$_3$ (*D*) (Wolf, 2004).

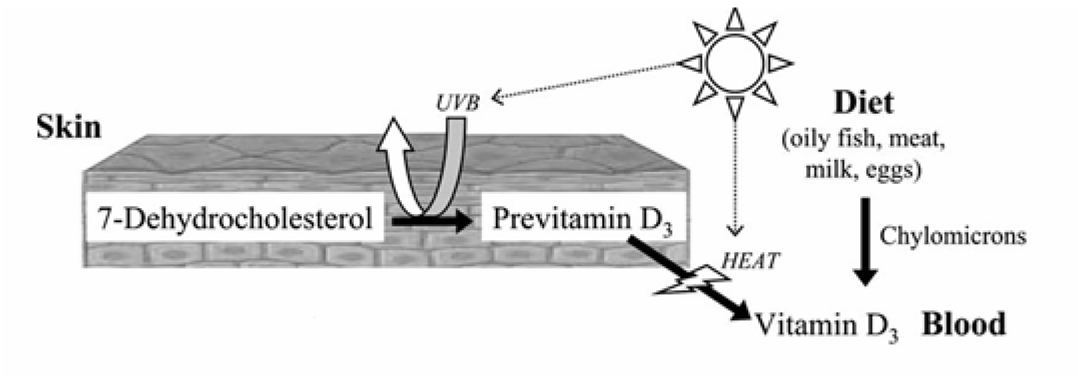


Figure 2: photochemical conversion of 7-dehydrocholesterol to previtamin D$_3$ (X.Palomer *et al*., 2008)

Vitamin D$_3$ is drawn into the dermal capillary bed by the vitamin D-binding protein, which has a low but effective affinity for it (Haddad *et al*., 1993). Vitamin D binds to vitamin D-binding protein (DBP) until it reaches its target tissue by means of the vitamin D receptors (VDR) (Holick, 2004). Intestinal absorption of vitamin D is mediated by bile salts and fatty acids in which vitamin D enters the plasma as a chylomicron/liproprotein complex through the intestinal-lymphatic system (Omdal *et al*., 2002).

Vitamin D_3 itself is biologically inert and requires two successive hydroxylations, one in the liver (on C25) and one in the kidney (on the α position of C1), to form its hormonally active metabolite, $1,25(OH)_2D_3$ (Mathieu *et al*., 2005). Orally ingested and endogenously formed vitamin D is transported to the liver where is it converted to 25-hydroxyvitamin D (25(OH)D) by 25-hydroxylase enzyme (Thacher and Clarke, 2011). There is no strong regulation of this step and there is no significant storage of 25(OH) D in the liver of mammals (Zittermann, 2003). The 25(OH)D is rapidly released by the liver into the blood, where it circulates with a biological half-life of approximately 12-19 day (Zittermann, 2003). In the kidney, 25(OH)D is enzymically converted to the vitamin D hormone 1,25-dihydroxyvitamin D (calcitriol) by 1 α-hydroxylase enzyme (Palomer *et al*., 2008). Renal synthesis of calcitriol is homeostatically controlled by parathyroid hormone (PTH) and synthesis of PTH is regulated by serum concentrations of calcium and phosphorus (Deluca, 2001).

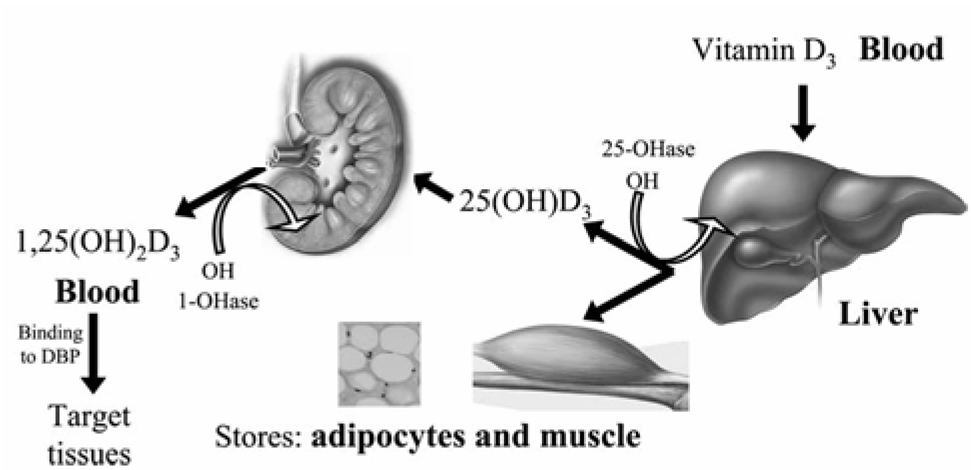


Figure 3: hydroxylation steps in the kidney and liver (Palomer *et al.*, 2008)

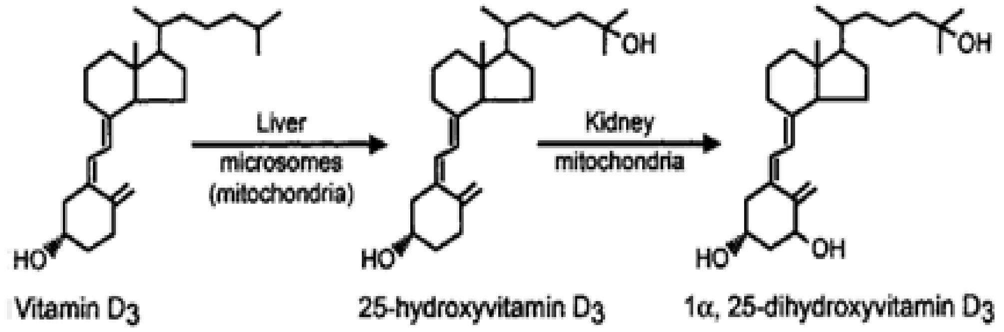


Figure 4: Metabolic activation of vitamin D_3 to its hormonal form, 1, $25(OH)_2$ D_3 (Deluca, 2004)

The major biological actions of vitamin D are maintenance of mineral homeostasis and regulation of bone remodelling (Zittermann, 2003). Active $1,25(OH)_2D_3$ stimulates intestinal calcium and phosphate absorption, bone calcium and phosphate resorption, and renal calcium and phosphate reabsorption, thus increasing calcium and phosphate ions in the blood (Haussler *et al*.,1998). Effects of $1,25(OH)_2D_3$ are mediated by the vitamin D receptor (VDR), a member of the nuclear receptor superfamily of transcriptional regulators, that is found in more than 30 different tissues, including islet cells of the pancreas, circulating monocytes, dendritic cells, and activated T cells (Nejentensev *et al*., 2004).

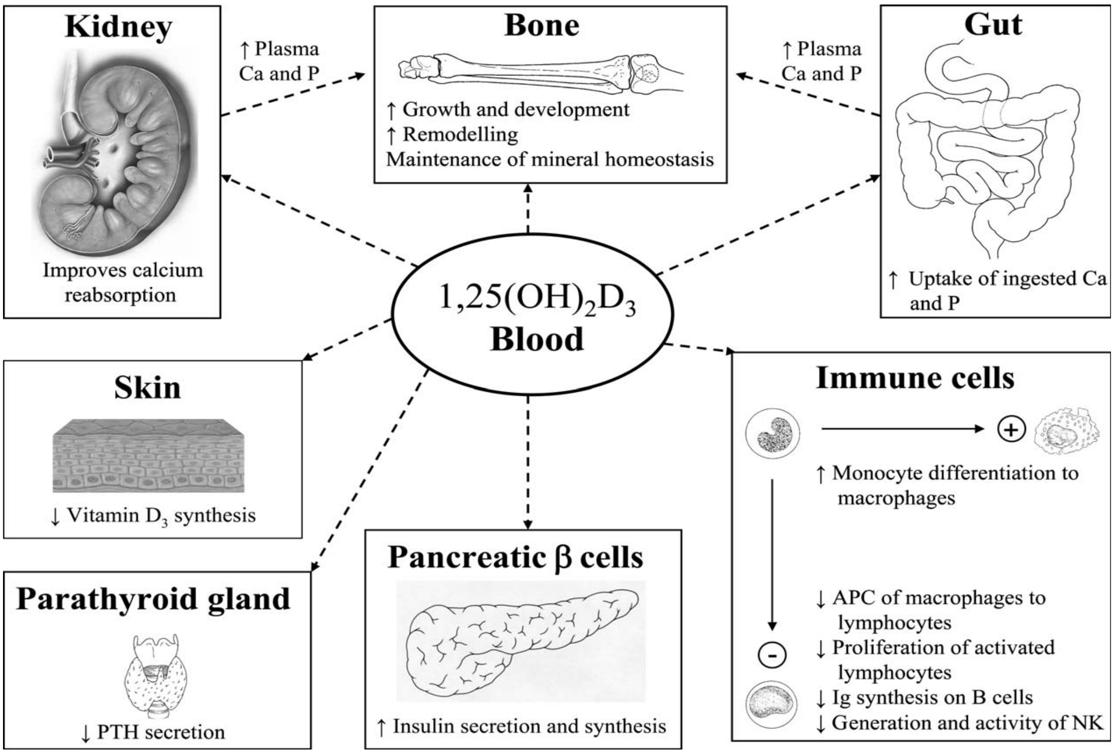


Figure 5: Major targets and actions of vitamin D_3 on peripheral tissues (Palomer *et al.*, 2008)

In addition to its effects on calcium homeostasis, 1, 25$(OH)_2$D regulates the functions of a wide variety of cells and tissues; indeed most normal cell types, and many tumour cells, express a VDR (Hart, 2004). The regulation of cell differentiation and proliferation is one key function, and the regulation of hormone secretion is another (Hart, 2004). Moreover, 1, 25$(OH)_2$D is a potent inhibitor of cellular growth, stimulator of insulin secretion, modulator of immune function and inhibitor of renin production (Holick, 2003). Tumor cells, that possess a VDR decrease their proliferative activity when exposed to 1, 25$(OH)_2$D (Garcion *et al.,* 2002, Hart, 2004). Of particular interest is the mounting evidence for the pivotal role of vitamin D in immune system regulation. Activated, but not resting, T- and B-lymphocytes, monocytes and macrophages respond to 1,25$(OH)_2$D, resulting in alteration of their immune functions (Hart, 2004). Additionally, 1,25$(OH)_2$D suppresses interleukin 2 production in activated T lymphocytes and monocytes, the leukocytes with the highest

phagocytosis capacity (Bemiss *et al*., 2002, Hart, 2004) and 1,25$(OH)_2D$ is able to induce the differentiation of monocytes into macrophages (Hart, 2004).

Table 1: Neoclassical 1, 25 $(OH)_2D_3$ target sites

Classification	Target tissue or cell	Specific effect	References
Immune system	Monocytes/macrophages and T-lymphocytes	Suppression of γ-iterferon and IL-1 through IL-6	Haussler *et al*., 1998
Central nervous system	Dorsal root ganglia, glial cells, and hippocampus	Production of nerve growth factor, neurotrophin-3, and leukemia inhibitory factor	Haussler *et al*., 1998
Epithelium	Epidermal skin/Keratinocyte hair follicle female reproductive tract mammary prostate colon lung	Differentiation Differentiation Uterine development ↓ cell growth ↓ cell growth ↓ cell growth Stimulate surfactant synthesis	Haussler *et al*., 1998
Endocrine system	Thyroid Pancreatic β-cell Ovary	↓ TSH action ↑ Insulin secretion Folliculogenesis, ↓ cell growth	Haussler *et al*., 1998
Muscle	Heart	↓ ANP (Arterial Natriuretic Peptide)	Haussler *et al*., 1998
Adipose	Adipocyte	↑ Lipoprotein lipase	Haussler *et al*., 1998
Many systems	Cancer Cell Line	↓ cell growth, apoptosis (↓ Bcl-2)	Haussler *et al*., 1998

1.3. MEASUREMENT OF VITAMIN D METABOLITES

The best approach for determining human vitamin D status is the assessment of serum 25(OH) D concentrations (Zittermann *et al.,* 2006). It reflects all sources of vitamin D exposure and has a half-life of 12-19 days; it is a comprehensive and stable indicator of vitamin D status (Song and Manson, 2010). For the measurement of circulating 25(OH)D, several analytical procedures such as radioimmunoassays (RIAs), enzyme-linked immunoassays (ELISAs), chemiluminescence assays, high-performance liquid chromatography (HPLC), and liquid chromatography tandem mass spectroscopy (LC-MS/MS) are available. The latter method being the reference analytical method (Zittrmann *et al.,*2009). However, cross-reactivity between 25(OH) D_3 and 25(OH) D_2 can vary between different test kits. Usually, circulating 25(OH) D_2 levels are negligible due to the low vitamin D_2 content of food (Zittrmann *et al.,* 2009). However, in countries where vitamin D_2 is used for food fortification and/or vitamin D_2 supplements are widely distributed, a test kit with similar cross-reactivity for 25(OH)D_3 and 25(OH)D_2 should be used. Alternatively, HPLC or LCMS/ MS can be applied, which allows separate detection of 25(OH)D_2 and 25(OH)D_3 (Lensmyer *et al.*, 2006) .

1.4. VITAMIN D DEFICIENCY

The most widely accepted definition of vitamin D deficiency is a serum level of 25(OH)D of less than 20 ng/ml; levels between 20 and 29 are considered insufficient, and levels of 30 ng/ml or more are considered sufficient (Batieha *et al.,*2011, Holick and Chen, 2008). This definition is based on the facts that: (1) serum 25(OH)D levels are inversely associated with parathyroid hormone levels until the former reaches 30-40 ng/ml, at which point parathyroid hormone levels begin to level off , and (2) intestinal calcium transport increases by 45-65% in women when 25(OH) D levels increased from an average of 20 ng/ml to an average of 32 ng/ml (Heany *et al*., 2003, Holick and Chen, 2008). vitamin D toxicity typically does not occur until 25(OH)D concentration is 150 ng/ml (Holick and Chen, 2008, Holick, 2007).

Vitamin D deficiency is currently so common and it is considered as a pandemic. Vitamin D deficiency or insufficiency affecting 1 billion people worldwide (Holick, 2007). Vitamin D deficiency is highly prevalent in the United States and Europe (Holick, 2007). About 30-50% of children and adults in Saudi Arabia, the United Arab Emirates, Australia, Turkey, India, and Lebanon had vitamin D deficiency with levels below 20 ng/ml (Holick, 2007). The major causes of vitamin D deficiency are; inadequate exposure to sunlight, very few food types naturally contain vitamin D, and food types that are fortified with vitamin D are often inadequate (Holick and Chen, 2008). Several other factors have been shown to be associated with vitamin D deficiency, including latitudes above 30° North and above 30° South, winter season, low altitude, skin pigmentation, use of sunscreen, indoor work, living in urban apartments, dressing styles covering most of the body, older age, and obesity (Batieha *et al.,*2011, Holick and Chen, 2008).

In Jordan, there is a high prevalence of low vitamin D status (25(OH)D of less than 30

ng/ml) in Jordanian women more than 18 years of age in contrast to a relatively low prevalence in men of the same age (Batieha *et al.,*2011).

Table 2: Prevalence of low vitamin D in the study population in Jordan (Batieha *et al.,*2011).

Variable	Prevalence of low 25(OH)D <30 ng/ml
Total	29.4 %
Age, years	
19-39	33.6 %
40-59	26.0 %
60+	25.2 %
Gender	
Male	51 %
Female	37.3 %

1.5. VITAMIN D RECEPTOR PROTEIN

Vitamin D functions through a single receptor known as vitamin D receptor (Deluca, 2004). The vitamin D receptor (VDR) is a member of the nuclear hormone receptor family, which also includes retinoid, thyroid hormone, and steroid hormone receptors (Jones *et al*., 1998). It has a variable N-terminal domain, a conserved DNA-binding domain (DBD), and a moderately conserved ligand-binding domain (Rochel *et al*., 2000). The human receptor is a 427-amino acid peptide. This receptor acts through vitamin D-responsive elements (VDREs), which are usually coiled for 1 kilobase of the start site of the target gene (Deluca, 2004). The VDREs are repeat sequences of 6 nucleotides separated by 3 nonspecified bases. The 5' arm of this sequence binds the retinoic acid X receptor and the 3' arm binds the VDR (Deluca, 2004).

The carboxyl terminal ligand biding domain of VDR interacts with a 43-residue amino-terminal domain in transcription factor II (TFIIB) (MacDonald *et al.*, 1995). The interaction with TFIIB showes selectivity for the ligand binding domain of VDR (MacDonald *et al.*, 1995). Together with the required retinoid X receptor (RXR), the VDR forms a heterodimer at the VDREs (Jurutka *et al.*, 2000). At the same time, it binds several other proteins required in the transcription complex and, most importantly, acquires an activator (Deluca, 2004). To date, at least three coactivators have been identified; steroid receptor coactivator (SARC1, -2, and -3) and VDR-interacting proteins (DRIP205) (Deluca, 2004, Oda *et al.*, 2006). There may be additional coactivators, and there may be selectivity among the coactivators with respect to which gene is being expressed (Deluca, 2004). Although there is little evidence for a co-repressor, it is thought that co-repressors will eventually be found for the VDR (Deluca, 2004). When the VDR interacts with the ligand, the repressor is no longer able to bind to the receptor, and the receptor changes conformation (Deluca, 2004). Phosphorylation on serine-205 occurs, and transcription is either initiated or suppressed, depending on the gene. (Deluca, 2004).

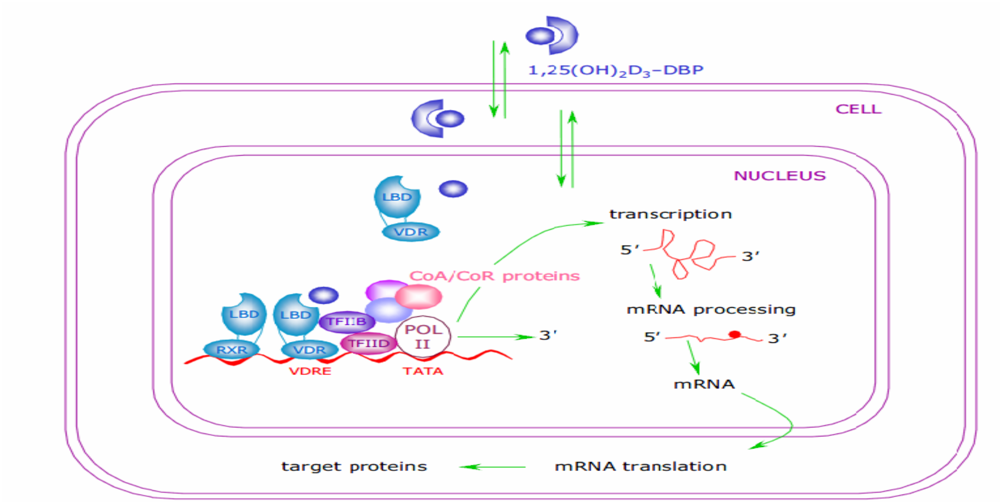


Figure 6: Genomic actions of 1α, 25-dihydroxyvitamin D_3 (Mathieu *et al.*, 2005)

1.6. VITAMIN D RECEPTOR GENE

The gene encoding VDR maps to the long arm of chromosome 12q13.1. It contains 14 exons, and spans approximately 100 kilobases of genomic DNA (Utterlinden *et al.,* 2004). Exons IA through IF encode the 5' untranslated region, exons II and III encode the DNA-binding domain, and exons IV-IX encode the ligand-binding region (Zumuda *et al*., 2000). The expression of the human VDR is under complex transcriptional control by multiple tissue-specific promoters (Zumuda *et al*., 2000). The N-terminal domain is configured into two zinc coordinated fingers responsible for DNA recognition and binding, whereas the C-terminal domain binds the 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] hormone (Hsieh *et al* ., 1991).

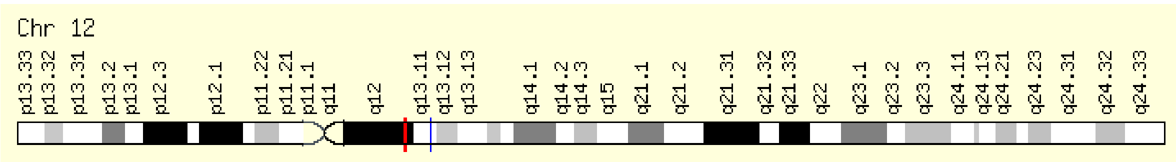


Figure 7: VDR genomic location

1.7. VITAMIN D RECEPTOR GENE POLYMORPHISMS

To date, more than 25 polymorphisms have been mapped to the VDR locus (Nejentsev *et al*., 2004). Four common single nucleotide polymorphisms (SNPs) in the VDR gene have been found; *Fok*I T_C (rs10735810), *Bsm*I A_G (rs1544410), *Apa*I G_T (rs7975232), and *Taq*I T_C (rs731236) (Nejentsev *et al*., 2004).

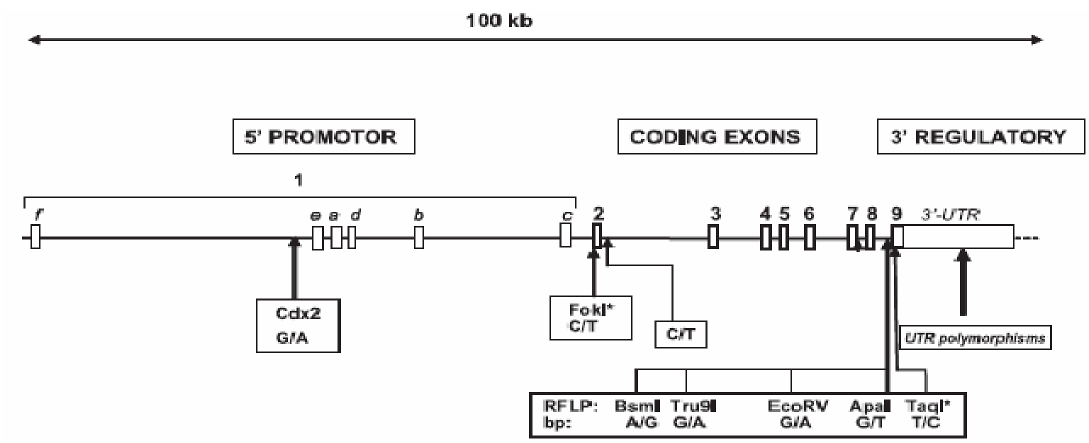


Figure 8: Exon–intron structure of the VDR gene and position of known polymorphisms. * indicates that these polymorphisms are in the coding sequence (Uitterlinden *et al*., 2004).

FokI polymorhism is a start codon polymorphism that results from a T/C polymorphism (ATG to ACG) at the first of two potential translation initiation sites in exon II. It has been defined using the *Fokl* restriction endonuclease (Zumuda *et al*., 2000, Uitterlinden *et al*., 2004). Individuals with the C allele (designated F) initiate translation at the second ATG site and lack the three NH2~ terminal amino acids of the full length VDR protein. In contrast, individuals with the T allele (designated f) initiate translation at the first ATG site and synthesize the full-length (427 amino acids) VDR protein (Zumuda *et al*., 2000). The (ff) genotype frequency was 4 %

among African Americans and 13-18 % among Asians and Caucasians. The 424 (FF) VDR interacts more efficiently with transcription factor TFIIB (Zumuda *et al*., 2000).

Bsml, Apal are restriction site polymorphisms that occur in the intron separating exons VIII and IX, resulting from A/G, G/T polymorphisms respectively (Zumuda *et al*., 2000). The *Bsml* bb genotype frequency was 2 % among Asians, 5 % among African Americans, and 17 % among Caucasians. The *Apal* AA genotype frequency is 9 % among Asians, 28 % among Caucasians, and 44 % among African Americans (Zumuda *et al*., 2000).

TaqI polymorphism results from T/C nucleotide substitution (ATT to ATC) leading to a silent change at codon 352 (isoleucine) in exon IX and is detected by the restriction enzyme *Taql (*Tylor *et al*., 1996). The frequency of *Taql* genotypes in these populations is similar to *Bsml* genotype frequencies (Zumuda *et al*., 2000).

The possible functional consequences of VDR alleles remain unclear. The *Apal* and *Bsml* variants are unlikely to have functional consequences, since both sites are located in the intron between exons VIII and IX and neither variant is near the intron-exon boundaries or known to produce splicing errors (Zumuda *et al*., 2000). The *Taql* polymorphism is also unlikely to directly affect VDR function, since both alleles code for isoleucine at amino acid 352 (Zumuda *et al*., 2000).

Other polymorphisms

Cdx2 is a G to A polymorphism found within a binding site of the intestinal-specific transcription factor Cdx-2 in the promoter region of the human vitamin D receptor (VDR) gene (VDR 1A promoter and VDR 1E promoter) (Fang *et al*., 2003). This polymorphism is present among Caucasians as well as other race groups. The 1E promoter region is suggested to play an important role in intestinal-specific transcription of the VDR gene (Fang *et al*., 2003). As the intestine is the predominant area for calcium absorption, the *Cdx2* site is thought to influence vitamin D regulation of calcium absorption (Fang *et al*., 2003). The A allele has been demonstrated to be more ‘‘active’’ than the G-allele by binding the Cdx2 transcription factor more strongly and by having more transcriptional activity (Arai *et al*., 2001). Thus, the A allele is thought to cause increased VDR expression in the intestine and, thereby, can increase the transcription of calcium transport proteins (such as calbindin 9K). This could thereby enhance the intestinal absorption of calcium (Fang *et al*., 2003).

A mononucleotide repeat [(A)n] polymorphism is a poly (A) that occurs in the 3' untranslated region of the VDR gene, which varies in length from 13 to 24 adenosines (Zumuda *et al*., 2000). The distribution of allele size is bimodal, such that individuals can be classified as having short (A13-A17) or long (A18-A24) alleles (Zumuda *et al*., 2000). The frequency of short alleles in one study was 5-10 % among Asians, 32 % among African Americans, and 41 % among Caucasians. The longest alleles (A23-A24) in a study were found only among African Americans, whereas the shortest allele (A13) was found only among Hispanics (Zumuda *et al*., 2000).

A novel **Tru 9 I** presents in intron 8 of VDR gene and it corresponds to a substitution of A for G at nucleotide at +443 pb from the end of exon 8 (Ye *et al*., 2000).

EcoVR is another restriction site polymorphism, detected by southern blot analysis of *EcoRV* digested genomic DNA probed with a VDR complementary DNA probe, in the intron separating exons VIII and IX, and it result from G/A substitution (Zumuda *et al*., 2000).

2. DIABETES MELLITUS

2.1. HISTORY

Diabetes mellitus symptoms were first described approximately 1500 BC (Jarrah *et al*., 2000). In 1921, Best and Banting extracted insulin from the pancreas, purified it and used it to treat diabetes in dog (Best and Scott, 1923).

2.2. DEFINITION

The term diabetes mellitus describes a metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism due to deficient action of insulin on target tissues resulting from insensitivity or lack of insulin (Albert *et al*., 1998). Diabetes mellitus may present with characteristic symptoms such as thirst, polyuria, blurring of vision, and weight loss (Albert *et al*., 1998).

2.3. CRITERIA FOR DIABETES MELLITUS DIAGNOSIS

The American Diabetes Association (ADA) defines normal glucose test as a fasting plasma glucose (FPG) of less than 100 mg/dl (less than 5.6 mmol/l), or two-hour post prandial plasma glucose in response to a 75 g oral glucose tolerance test of less than 140 mg/dl (less than 7.8 mmol/l). Diabetes is defined according to The American Diabetes Association (ADA) as a fasting plasma glucose (FPG) of 126 mg/dl or more (of 7.0 mmol/l or more) or two-hour post prandial plasma glucose of 200 mg/dl or more (of 11.1mmol/l or more) during an oral glucose tolerance test (OGTT) (Abujbara *et al*., 2009).

Because HbA1c level reflects average of blood glucose over 2-3 months and has strong predictive value for diabetes complications ADA recommended the use of HbA1c test for diagnosis of diabetes and then as a part of continuing care (American Diabetes Association, 2011).

There are three ways of diagnosing diabetes mellitus (American Diabetes Association, 2011):

(a) HbA1c level of 6.5 % or more.

(b) Fasting plasma glucose of 126 mg/dl or more (of 7mmol/l mg/dl ore more).

(c) Two-hour post prandial plasma glucose of 200 mg/dl or more(of 11.1mmol/l mg/dl or more).

(d) Symptoms (e.g. polyuria, polydispia, weight loss, blurred vision) plus random blood glucose of 200 mg/dl or more (of 11.1mmol/l mg/dl or more).

There is also a metabolic state between diabetes and normal glucose homeostasis. This is known as impaired glucose regulation and defined as either abnormal fasting glucose (impaired fasting glucose (IFG)) which is defined by an elevated fasting

plasma glucose (FPG) concentration (of 100 mg/dl or more and less than 126 mg/dl) or abnormal post prandial glucose levels (impaired glucose tolerance (IGT)) which is defined by an elevated two-hour plasma glucose concentration (of 140 mg/dl or more and less than 200 mg/dl) after a 75-g glucose load on the oral glucose tolerance test (OGTT) in the presence of a fasting plasma glucose FPG concentration of less than 126 mg/dl (Ytes *at el*., 2006, American Diabetes Association, 2011).

Table 3: Diagnostic thresholds for diabetes (American Diabetes Association, 2011)

	Test	
Category	FPG	2-h PG
Normal	<100 mg/dl (<5.6 mmol/l)	<140 mg/dl (<7.8 mmol/l)
IFG	100-125 mg/dl(5.6-6.9 mmol/l)	_
IGT	_	140-199 mg/dl (7.8-11.0 mmol/l)
Diabetes	≥126 mg/dl (≥7.0 mmol/l)	≥200 mg/dl (≥11.1 mmol/l)

2.4. Types of diabetes mellitus

Types of diabetes mellitus are classified according to the ADA into:

1. Type 1 diabetes mellitus

Appears in children and youth due to autoimmune destruction of the β cells of the islets of langerhans in the pancreas, and lead to absolute deficiency of the insulin hormone (Jarrah *et al*., 2000).

2. Type 2 diabetes mellitus

The most common form of diabetes, affecting older subjects, mainly the obese, due to abnormalities at the receptor level resulting in insulin resistance, where there is relative insulin deficiency due to partial β cell failure (Jarrah *et al*., 2000).

3. Gestational diabetes mellitus: when diabetes is diagnosed for the first time during pregnancy (Jarrah *et al*., 2000).

4. Other specific types: these are less common types of diabetes, those in which the underlying defect or disease process can be identified in a relatively specific manner. These types include a group of diabetes mellitus cases with monogenetic defects in β cell function (e.g. maturity onset diabetes of the young (MODY), genetic defect in insulin action (e.g. lipoatrophic diabetes), disease of the pancreas, endocrinopathies with glucose intolerance (e.g. Cushing's syndrome), drug induced diabetes mellitus, infections and syndromes associated with glucose intolerance like Prader-Willi Syndrome (Jarrah *et al*., 2000).

2.5. Complications of diabetes

Without proper insulin production and action, sugar remains in the blood, leading to chronic hyperglycaemia. This can result in short and long-term complications, many of which, if not prevented and left untreated, can be fatal. All have the potential to reduce the quality of life of people with diabetes and their families (International Diabetes Federation, 2006).

The most common long-term complications are:

1 **Cardiovascular disease**: affects the heart and blood vessels and may cause fatal complications such as coronary heart disease (leading to a heart attack) and stroke. People with diabetes are two to four times more likely to develop cardiovascular disease than people without diabetes (Selvin *et al*., 2004, International Diabetes Federation, 2011).

2 **Diabetic neuropathy**: damage to nerve fibers primarily affecting the legs and feet. Foot ulcers are common symptoms. Infections in these wounds may

ultimately result in amputation of the foot and lower leg. It is estimated that up to 70% of all lower-limb amputations are related to diabetes (International Diabetes Federation, 2011).

3 **Diabetic nephropathy**: may result in total renal failure and in the need for dialysis or kidney transplant. Diabetes is the leading cause of renal failure in the developed world and accounts for approximately 35% to 40% of new cases of end stage renal disease (ESRD) each year (Sheetz and King, 2002, International Diabetes Federation, 2011).

4 **Diabetic retinopathy**: damage to the retina of the eye which can lead to vision loss. The incidence of blindness is 25 times higher in people with diabetes than in the general population (Fong *et al*., 2004, International Diabetes Federation, 2011).

Short-term complications: These include; Low blood sugar, Ketoacidosis, Lactic acidosis and bacterial/fungal infections (International Diabetes Federation, 2006).

2.6. PREVALENCE OF DIABETES

The number of people with diabetes is increasing due to population growth, aging, urbanization, and increasing prevalence of obesity and physical inactivity (Wild *et al*., 2004). The prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% in 2000 and will be 4.4% in 2030 (Wild *et al*., 2004). The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 (Wild *et al*., 2004, Pittas and Dawson-Hughes, 2010). The urban population in developing countries is projected to double between 2000 and 2030. The most important demographic change to diabetes prevalence across the world appears to be the increase in the proportion of people above 65 years of age (Wild *et al*., 2004). In another study, the number of people with diabetes is expected to almost double in the

next two decades, increasing from 194 million in 2003 to 380 million in 2025 (Abujbara *et al*., 2008). In Jordan, compared to the 1994 survey, there was a significant increase in the prevalence of diabetes by 31.5% and the prevalence of type 2 diabetes and IFG is high and is increasing (Ajlouni *et al*., 2008).

Table 4: Prevalence estimates of diabetes 2010 in Arab countries (Diabetes Atlas, International Diabetes Federation, 2010)

Country	Prevalence of DM 2010 (%)
Bahrain	14.4
Egypt	10.4
Iraq	7.8
Jordan	**7.5**
Kuwait	10.8
Lebanon	7.7
Libya	7.5
Morocco	7.6
Oman	11.1
Palestine Territory, occupied	6.4
Qatar	13.3
Saudi Arabia	13.6
Sudan	3.3
Syria	8.3
Tunisia	8.5
United Arab Emirate	12.2
Yemen	2.5

Table 5: Prevalence estimates of diabetes 2010 in other countries (Diabetes Atlas, International Diabetes Federation, 2010)

Country	Prevalence of DM 2010 (%)
United State of America	12.3
Germany	12
Canada	11.6
France	9.4
Italy	8.8
Spain	8.7
India	7.1
Bangladesh	6.1
Iran	6.1
United Kingdom	4.9

2.7. VITAMIN D RECEPTOR POLYMORPHISMS AND DIABETES

Several epidemiological studies suggest that there is biological evidence implicating a potential influence of vitamin D on glucose homeostasis (Mathieu *et al.,* 2005). The inferences for the manifold roles of vitamin D include; the presence of specific vitamin D receptors (VDRs) on pancreatic β-cells and the expression of 1-α-hydroxylase enzyme in pancreatic β-cells, which catalyzes the conversion of 25(OH)D to 1, 25dihydroxyvitamin D, the presence of a vitamin D response element in the human insulin gene promoter and the presence of VDR in skeletal muscle (Maestro *et al*., 2003, Alvarez and Ashraf, 2010). In addition, 1, 25$(OH)_2$D directly activates transcription of the human insulin receptor gene, activates peroxisome proliferator activator receptor-δ, stimulates the expression of insulin receptor, and enhances insulin-mediated glucose transport in vitro (Alvarez and Ashraf, 2010).

Vitamin D deficiency results in elevated parathyroid hormone (PTH), which in turn is known to elevate intracellular calcium. Sustained elevations of intracellular calcium may inhibit insulin-target cells from sensing the brisk intracellular calcium fluxes necessary for insulin action, such as glucose transport (Worrall and Olefsky, 2002). Pancreatic β-cell also depends on an acute intracellular calcium increase for insulin secretion, which may also be attenuated with elevated cytosolic calcium (Alvarez and Ashraf, 2010). Another possible mechanism is that elevated intracellular calcium enhances calmodulin binding to insulin receptor substrate-1 (IRS-1), which interferes with insulin-stimulated tyrosine phosphorylation and PI3-kinase activation (Alvarez and Ashraf, 2010).

Several studies have demonstrated a link between VDR gene polymorphisms and type 2 diabetes, although the findings differ from one population to another. Hitman *et al.* (1998) showed an association between the ApaI polymorphism (homozygosis for the A allele) and lower insulin secretion in a healthy Bangladeshi Asian population at risk of type 2 diabetes living in London who, have a high prevalence of vitamin D deficiency. Speer *et al.* (2001) reported that patients with diabetes and obesity with the BB genotype of the BsmI allele in the VDR gene presented higher levels of postprandial serum C-peptide, which indicates that the polymorphisms of the VDR gene might play a role in the pathogenesis of type 2 diabetes by influencing the secretory capacity of β-cells. It has been reported that the same VDR B allele, which predisposes to altered calcium absorption, elevated PTH and type 2 diabetes mellitus, is associated with elevated fasting glucose in healthy young men long before the onset of type 2 diabetes (Ortlepp *et al*., 2003). In another study, three single nucleotide polymorphisms were examined (BsmI, ApaI, TaqI), where the study found that the distributions of genotype frequencies of ApaI, BsmI, and TaqI polymorphisms did not differ between persons with newly diagnosed type 2 diabetes and without diabetes, but ApaI polymorphism may be associated with glucose intolerance and fasting plasma glucose and BsmI polymorphism may be associated with insulin resistance in a nondiabetic Caucasian population (Oh and Barret-Connor, 2002). Ogunkolade *et al.* (2002) also showed a positive association between the TaqI (genotype TT) and the BsmI (genotype bb) polymorphisms with reduced insulin secretory capacity in the Bangladeshi adults. It has also been reported that in subjects with 25(OH)D_3 insufficiencies, TaqI polymorphism is a determinant of insulin secretion (Ogunkolade *et al*., 2002). In another study in a healthy glucose-tolerant Caucasian population, subjects with the homozygous FF genotype showed increased insulin sensitivity

compared with those with the f allele, although no influence on β cell function was observed (Chiu *et al*., 2001). Ye *et al*. (2000), who studied four different polymorphisms of the VDR gene (three in intron 8, BsmI, Tru9I and ApaI, and one in exon 9, TaqI), concluded that this was not a major predisposing gene for type 2 diabetes in French Caucasians. However, polymorphism in VDR gene was associated with susceptibility to obesity in subjects with early-onset T2DM. The same authors suggested that this effect could be related to a direct action of vitamin D on adipocyte differentiation and metabolism or to an indirect modulation of insulin secretion. Dilmec *et al*. (2010) who examined two single nucleotide polymorphisms (ApaI, TaqI) of the VDR gene study did not provide evidence for the association between the two examined VDR polymorphisms and type 2 diabetes mellitus in Turkish population. In another study that examined one SNP (BsmI), it was found that there is a limited influence of VDR gene polymorphism on the bone mineral density (BMD) of patients with type 2 diabetes mellitus (Ma *et al*., 2001).

PATIENTS, MATERIALS AND METHODS

1. STUDY POPULATION.

1.1. Patients.

A total of (200) adult diabetic patients type 2 attending at the diabetes clinics in the Jordan University Hospital (JUH) and the National Center for Diabetes, Endocrinology and Genetics (NCDEG) in the period from August 2010 to September 2010 were included in the study.

1.2. Controls.

A total of (200) nondiabetic and nonobese adults were included in the study and served as a control group as mentioned in results section.

1.3. Data collection

An informed consent was obtained from all patients and controls enrolled in the study as shown in appendix (A). Results of standard biochemical laboratory tests like (Fasting blood sugar, HbA1C, urea, creatinine, Na, K and Ca) were obtained from patient files. Vitamin D levels were obtained from patient files Vitamin D level and were determined using raidioimmonuassay (RIA) (vitamin D free DIA source kit) and enzyme-linked immunoassays (ELISAs assay) (immunodiagnostic system (IDS) kit). Body mass index was calculated from the height and weight of patients according to body mass index equation (BMI = weight (kg) / (height (m))^2). Diabetic patients were classified into obese, overweight and nonobese according to world health organization classification. Diabetic patients that have (18.5-24.5 kg/m^2) BMI range were considered nonobese, diabetic patients that have (25.0-29.9 kg/m^2) BMI range were considered overweight,

and diabetic patients that have BMI value of more than 30 kg/m^2 were considered obese. Obese and overweight diabetic patients were put in one category.

Diabetic patients were classified into normotensive and hypertensive or into diabetic patients with coronary artery disease and without coronary artery disease according to their medical files.

1.4. Collection of blood samples:

Four milliliters of venous blood were drawn from each patient and control subjects into tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The blood samples were immediately processed or stored at 4 °C until processed.

2. MATERIALS.

2.1. Reagents:

- **Wizard® Genomic DNA Purification Kit** (Promega, USA)
- **Absolute Ethanol 96%** (Ginaland Chemical Company, UK).
- **Isopropanol** (BioBasic Inc, Canada).
- **GoTaq® DNA Polymerase** (Promega, USA)
- **$MgCl_2$,** molecular biology grade (Promega, USA): supplied as 25mM $MgCl_2$ solution.
- **Nuclease free-water (NFW)** (Promega, USA).
- **Ethidium Bromide;** molecular biology grade (Electron, England).
- **1.5 ml microcentrifuge tubes (**Griener, Germany**).**
- **100-bp DNA Ladder** (Fermentas, EU).
- **Agarose powder** (Lonza, USA).

- **Primers** (Invitrogen, USA): The primers were used from previously published sequences (Vupputuri *et al*., 2006, Morrison *et al*., 1994)) shown in Table 6 .

- **BsmI, ApaI, TaqI Restriction Enzymes** ((New England Biolabs, USA)

Table 6: Primers' sequence used for detection of VDR gene polymorphisms

Primers	Primers sequence	No. of (bp)*
BsmI Forward	(5'CAACCAAGACTACAAGTACCGCGTCAGTGA3')	30
BsmI Reverse	(5'AACCAGCGGGAAGAGGTCAAGGG3')	23
TaqI Forward	(5'CAGAGCATGGACAGGGAGC3')	19
TaqI Reverse	(5'AGGAGAGGCAGCGGTACTG3')	19

*Base pair

2.2. Equipment:

- **PTC- 100 Peltier Thermal Cycler** (MJ Research, USA).
- **Electrophoresis power supply** (Apelex, France).
- **Electrophoresis Chamber or Tank** (Cleaver, USA).
- **Balance** (Denver Instrument Company, USA).
- **Smart Spec^{TM} Plus spectrophotometer** (Bio-Rad, USA).

- **AF – 10 Distillizer** (Elga, UK).
- **Microwave** (Panasonic, USA).
- **Micro-120 microcentrifuge (**Hettich zentrefugen, Germany**).**
- **Centrifuge modle :PLC-025 (**Gemmy industrial company, Taiwan**).**
- **Gel Doc- It^{TM} Imaging system** (UVP, UK).
- **0.5-10 micropipette** (witeg, Germany).
- **10-100 micropipette** (Dragon MED, China).
- **100-1000 micropipette** (HTL, Poland).
- **Autoclave (**JP Selecta, Italy**).**
- **Ultra-violet transilluminator** (UVP, USA)
- **PCR tubes with flat caps** (Bio-Rad, USA)

3. METHODS

3.1. DNA Extraction:

Genomic deoxyribonucleic acid (DNA) was isolated from peripheral leukocytes using a DNA extraction kit as described by the kit protocol.

Cell lysis: a volume of 300 µl of buffy coat were placed into 1.5 ml Eppendrof tubes containing 900 µl red blood cells (RBC) lysing solution and incubated for 10 minutes at room temperature. During the incubation period, the tubes were mixed gently by inversion for at least 2-3 times. After incubation, the tubes were centrifuged for 20 seconds at 13,000 X g. The supernatant was removed from the tubes by a pipette leaving behind the visible white blood cells (WBC) pellet and about 10-20 µl of the residual RBC's lysing liquid. Pellets of WBC's were then resuspended and well mixed in the residual liquid.

Protein precipitation: A volume of 300 µl nuclei lysing solution was added to the cell lysate and mixed by pipetting, and a volume of 100 µl protein precipitation solution was added to the tube and mixed uniformly by vortexing and the tubes were centrifuged for 3 minutes at 13,000 X g. The precipitated protein formed a tight, dark brown pellet.

DNA precipitation: The supernatant containing the DNA (leaving behind precipitated protein pellet) was transferred into a clean 1.5 ml Eppendrof tube containing 300 µl absolute isopropanol. The tubes contents were mixed gently by inversion. The DNA was visible as a thread-like structure and as a small white pellet after the centrifugation of the sample for 3 minutes at 13,000 X g. The supernatant was decanted and a volume of 300 µl of 70% ethanol was added to the tubes and mixed gently by inversion. The

tubes were centrifuged for 3 minutes at 13,000 X g and were drained on a clean absorbent paper to allow them to air dry.

DNA hydration: A volume of 50-100 µl DNA hydration solution was added to each tube and mixed and stored at -20 °C until they were analyzed. The DNA concentration in the aqueous phase was measured to determine absorbance at a wavelength of 260 nm using Gene Quant II spectrophotometer.

3.2. DNA Quantitation by UV spectrophotometery:

Spectrophotometery was used to estimate the concentration of extracted genomic DNA. A total volume of 100 µl containing 4µl DNA and 96 µl distilled water was needed to measure DNA concentration at wave lengths of 260 nm and 280 nm against a blank of distilled water. The purity of DNA was estimated using the ratio between OD260/OD280. The correct ratio should be between 1.65-1.85.

4. POLYMERASE CHAIN REACTION.

4.1. Amplification of negative control:

Sterile deionized water was used as a negative control to exclude any contamination of PCR components or the master mix itself. A volume of 18 µl of deionized water was added to 29 µl master mix.

4.2. Optimal concentrations and conditions for the PCR.

Extracted DNA was amplified by a PCR Thermal Cycler. Amplification was performed according to previously described protocol (Chang *et a*l., 2000, Vupputuri *et al.*, 2006)). Briefly, a total volume of 50 µl containing 3 µl genomic DNA, 1 µl of each primer, 25 µl master mix and 2 µl of MgCl2. The final PCR mixture volume was completed to 50 µl by adding nuclease free water (18 µl) as shown in Table 7.

Reaction was performed for 35 cycles, each consisting of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds. Predenaturation and final extension were performed at 94°C for 5 minutes and 72°C for 5 minutes respectively as shown in Table 8.

Table 7: The volumes used in PCR reaction.

PCR reaction mixture	Volumes	Concentrations
Master mix: Buffer: dNTPs*: (10µM) Taq polymerase	25 µl of 2X manufactured Master mix	1x
F*-Primer: R*-Primer:	1 µl 1 µl	20 pmol/ µl 20 pmol/ µl
DNA	3 µl	
Nuclease free water	18 µl	
Total:	50 µl	

*dNTPs: deoxyribonucleotide triphosphate, F: forward, R: reverse

Table 8: Cycling conditions for the PCR.

Steps	Duration	Temperature (°C)
Pre-denaturation	5 min*	94
Denaturation	30 sec*	94
Annealing	30 sec	60
Extension	30 sec	72
Final extension	5 min	72

*Min=minute, Sec=second. Numbers of cycle=35.

5. AGAROSE GEL ELECTROPHORESIS

The 2% agarose gel was prepared as follows:

Agarose (2 g) was added to 100 ml of 1X TBE buffer (Appendix C) with gentle shaking. After completely dissolving of the agarose by boiling, 30 µl of (2.5mg/ml) ethidium bromide was added. The agarose was then poured into the electrophoresis cell and left to solidify at room temperature for 15 minutes. After removal of combs and gates, the 1X TBE buffer was added to the electrophoresis cell, and the volume of 7 µl of PCR product were loaded in the gel which was run for 25 minutes at 120 volts.

6. RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

6.1. Optimal concentrations and conditions for the RFLP

The PCR product of BsmI primers was digested with BsmI endonuclease enzyme at 65°C overnight. The PCR product of TaqI primers was digested with TaqI endonuclease enzyme at 65°C overnight and ApaI endonucleases enzymes at 37°C overnight. Briefly, a total volume of 25 µl containing 2.5 µl buffers, 1 µl enzyme, 1.5 µl nuclease free water and 20 µl PCR product was used for BsmI enzyme and 2.5 µl buffer, 1 µl enzyme, 0.25 µl BSA, 6.25 µl nuclease free water and 15 µl of PCR product was used for TaqI and ApaI enzymes as shown in Table 9.

Table 9: The volumes used in RFLP

RFLP Mixture	Volumes for BsmI	Volumes for TaqI and ApaI
Buffer Enzyme BSA	2.5 µl 1 µl (10 U/µl) _	2.5 µl 1 µl (TaqI =20 U/ µl, ApaI =10 U/µl) 0.25 µl
PCR product	20 µl	15 µl
Nuclease free water	1.5 µl	6.25 µl
Total	25 µl	25 µl

7. AGAROSE GEL ELECTROPHORESIS

2.5% agarose gel was prepared by adding (2.5g) Agarose to 100 ml of 1X TBE buffer (Appendix C) with gentle shaking and 3% agarose gel was prepared by adding (3g) Agarose to 100 ml of 1X TBE buffer with gentle shaking. After completely dissolving of the agarose by boiling, 30 µl of (2.5mg/ml) ethidium bromide was added. The agarose was then poured into the electrophoresis cell and left to solidify at room temperature for at least 15 minutes. After the combs and gates were removed, 1X TBE buffer was added to the electrophoresis cell, and 15 µl of RFLP product was loaded in the gel and allowed to run for 25 minutes at 120 volts.

8. STATISTICAL ANALYSIS

Data were entered and analyzed using Statistical Package of Social Sciences (SPSS) version 16. Continuous data are presented as mean ± SD (Standard deviation) and qualitative data are presented as frequencies. Descriptive statistical analysis was used for demographic and baseline information. The association between BsmI, TaqI and ApaI genetic polymorphisms and type 2 diabetes risk (T2DM) was analyzed by

calculating the crude odds ratio (OR) and 95% confidence interval (95% CI) using binary logistic regression. Frequencies and proportions were found by Chi-square test. The same calculations were used to analyze the association between BsmI, TaqI and ApaI genetic polymorphisms and hypertension, obesity and coronary artery disease risk among diabetic patients. The association between continuous variables (fasting blood sugar, HbA1c %, systolic and diastolic blood pressure) and BsmI, TaqI and ApaI genetic polymorphisms was analyzed using binary logistic regression. Mean and standard deviation were found by the independent-sample t-test. The same calculations were used to analyze the association between different variables and vitamin D levels. A p-value < 0.05 was considered as statistically significant.

Allele frequency was calculated according to the following equation:

Number of patients with AA allele (two alleles of A) = 78

Number of patients with AG allele (one allele of A and one allele of G) = 58

Number of patients with GG alleles (two alleles of G) = 64

So

Number of A alleles = 78 * 2 + 58 * 1 = 214

Number of G alleles = 64 * 2 + 58 * 1 = 186

A allele frequency = 214/400 = 0.535

G allele frequency = 186/400 = 0.465

In the same way, the calculations are done for the other alleles for patients and controls.

RESULTS

4. Characteristics of study population

In this study, the frequencies of BsmI, TaqI and ApaI polymorphisms of the VDR gene among diabetic patients attending the JUH and the National Center for Diabetes, Endocrinology and Genetics (NCDEG) was examined and compared in order to determine a possible association between these polymorphisms and susceptibility to diabetes. The association between these polymorphisms and disease variables and complications was also examined.

Two hundred Jordanian adult diabetic patients type 2 and two hundred age and sex matched Jordanian nonobese and nondiabetic subjects were included in the study as shown in Table 10. The age of patients did not statistically differ from controls (Mean age of patients was (57.22±11) years and mean age of controls was (55.08±12) years). The number of females and males of patients (with a female to male ratio (52/48)) did not statistically differ from controls (with a female to male ratio (56/44)). There is a significant difference between the body mass index mean for patients (30.27±4.6) and, the body mass index mean for controls (23.12±1.3). Both PCR and RFLP were performed to detect these polymorphisms at the VDR locus.

Table 10: Demographic characteristics of patients and controls

Demographic parameter	**200-diabetic patients N (%)**		**200-control subjects N (%)**	
Gender	**Male**	**Female**	**Male**	**Female**
	104 (52)%	96 (48)%	112 (56)%	88 (44)%
Age (years) Mean ± SD* Range	57.01±11 31-81	57.43±11 30-81	57.65±12 30-79	53.07±11 30-79
Age (years) Mean ± SD Range	57.22±11 30-81		55.08±12 30-79	
BMI*	30.27±4.6		23.12±1.3	

BMI: body mass index, SD: Standard Deviation.

Diabetic patients of this study had fasting blood sugar levels above the normal range (mean of FBS=168±72 mg/dl) and elevated HbA1c% with a mean was 7.7±1.5%. Systolic blood pressure at upper limits (131±21 mmHg), and diastolic blood pressure was within normal range (76±12 mmHg). The mean of sodium, potassium, calcium, urea and createnine levels were within normal ranges ; 140±3 mmol/l, 4.46±0.44 mmol/l, 9.4±0.5 mmol/l, 36.37±17.79 mg/dl, 0.79±0.36 mg/dl respectively as shown in Table 11.

Table 11: Laboratory and clinical findings of the diabetic patients

Clinical feature	Mean±SD	Normal range
FBS*(mg/dl)	168±72	70-100 mg/dl
HbA1c%	7.7±1.5	4.2-6.5 %
Systolic blood pressure (mmHg)	131±21	< 120 mmHg
Diastolic blood pressure (mmHg)	76±12	< 80 mmHg
Sodium (mmol/l)	140±3	135-152 mmol/l
Potassium (mmol/l)	4.46±0.44	3.5-5.5 mmol/l
Calcium (mmol/l)	9.4±0.5	8.5-10.5mg/dl
Urea (mg/dl)	36.37±17.79	15-46 mg/dl
Createnine (mg/dl)	0.79±0.36	0.6-1.36 mg/dl

*FBS: Fasting blood sugar.

Patients (28±22) had a higher vitamin D levels than controls (15±9) (p=0.000, OR= 1.064, 95%CI= (1.045-1.084)). The prevalence of vitamin D deficiency (<20 ng/ml) was significantly higher in controls than in patients (p= 0.000). Conversely, the prevalence of vitamin D insufficiency (20-29.9 ng/ml) and vitamin D sufficiency (≥ 30 ng/ml) was significantly higher in male patients than in male controls as shown as shown in Table 12.

Table 12: vitamin D levels in patients and controls

Vitamin D level	Patients (n=200)	Control (n=200)	*p*-value OR, (95%CI)
25(OH)D <20 (ng/ml)	91 (45.5%)	151 (75.5%)	0.000 1, reference.
25(OH)D 20- 29.9(ng/ml)	44 (22.0%)	38 (19%)	0.011 1.202 (0.739-1.956)
25(OH)D ≥30 (ng/ml)	65 (32.5%)	11 (5.5%)	0.000 9.805 (4.918-19.547)

Male patients (37±21) had a higher vitamin D levels than male controls (18±10) (p=0.000, OR= 1.086, 95%CI= (1.055-1.117)). The prevalence of vitamin D deficiency (<20 ng/ml) was significantly higher in male controls than in male patients (p= 0.000, OR = 1 (reference)). The prevalence of vitamin D insufficiency (20-29.9 ng/ml) and vitamin D sufficiency (≥ 30 ng/ml) was significantly higher in male patients than in male controls as shown in Table 13.

Table 13: Vitamin D levels among males

Vitamin D level	Male (Patients) (n=96)	Male (Controls) (n=88)	*p*-value OR, (95%CI)
25(OH)D <20 (ng/ml)	24 (25%)	61 (69.3%)	0.000 1, reference
25(OH)D 20- 29.9(ng/ml)	22 (22.9%)	19 (21.6%)	0.006 2.943 (1.357-6.385)
25(OH)D ≥30 (ng/ml)	50 (52.1%)	8 (9.1%)	0.000 15.885 (6.568-38.421)

Female patients (20±20) had a higher vitamin D levels than female controls (12±8) (p=0.000, OR= 1.056, 95%CI= (1.025-1.088)). The prevalence of vitamin D deficiency (<20 ng/ml) was significantly higher in female controls than in female patients (p= 0.009, OR = 1 (reference)). The prevalence of vitamin D sufficiency (≥ 30 ng/ml) was significantly higher in female patients than in female controls (p= 0.004, OR = 6.716, 95% CI = (1.869-24.140)). No significant difference was observed between female patients and female controls in the term of insufficient levels of vitamin D (20-29.9) (ng/ml) as shown in Table 14.

Table 14: **Vitamin D levels among females**

Vitamin D level	Female (Patients) (n=104)	Female (controls) (n=112)	p-value OR, (95%CI)
25(OH)D <20 (ng/ml)	67 (64.4%)	90 (80.4%)	0.009 1, reference
25(OH)D 20- 29.9(ng/ml)	22 (21.2%)	19 (17.0%)	0.210 1.555 (0.780-3.103)
25(OH)D ≥30 (ng/ml)	15 (14.4%)	3 (2.7%)	0.004 6.716 (1.869-24.140)

No significant difference was observed in the FBS levels or HbA1c according to vitamin D levels (Table 15).

Table 15: mean of FBS and HbA1c according to vitamin D level

Vitamin D levels	FBS (mg/dl)	HbA1c%
25(OH)D ≤29.9 (ng/ml) mean±SD	173.23±78	7.7±1.6
25(OH)D ≥30 (ng/ml) mean±SD	156.82±58.20	7.7±1.5
***p*-value**	0.137	0.898

*FBS: fasting blood sugar

2. Vitamin D level and disease variables and complications

In terms of vitamin D levels, no significant difference was observed between diabetic patients with hypertension and diabetic patients without hypertension, between obese diabetic patients and non obese diabetic patients, and between diabetic patients with coronary artery disease and diabetic patients without coronary artery disease as shown in Tables 16, 17, and 18.

Table 16: Vitamin D levels among diabetic patients with and without hypertension.

Vitamin D level	hypertensive diabetic patients (n=156)	normotensive diabetic patients (n=44)	*p*-value OR, (95%CI)
25(OH)D ≤29.9 (ng/ml)	106 (67.9%)	29 (65.9%)	0.799
25(OH)D ≥30 (ng/ml)	50 (32.1%)	15 (34.1%)	0.912 (0.449-1.852)

Table 17: Vitamin D levels among obese diabetic patients and non obese diabetic patients

Vitamin D level	Obese or overweight diabetic patients (n=169)	Nonobese diabetic patients (n=31)	*p*-value OR, (95%CI)
25(OH)D ≤29.9 (ng/ml)	114 (67.5%)	21 (67.7%)	0.975
25(OH)D ≥30 (ng/ml)	55 (32.5%)	10 (32.3%)	0.987 (0.435-2.239)

Table 18: Vitamin D levels among diabetic patients with and without coronary artery disease.

Vitamin D level	CAD*-diabetic patients (n=33)	diabetic patients without CAD (n=167)	*p*-value OR, (95%CI
25(OH)D ≤29.9 (ng/ml)	19 (57.6%)	116 (69.5%)	0.186
25(OH)D ≥30 (ng/ml)	14 (42.4%)	51 (30.5%)	1.676 (0.780-3.601)

* CAD: coronary artery disease

3. Genotyping

3.1. Interpretation of the results.

The PCR revealed an 822-bp product when using BsmI primers, and 454-bp product when using TaqI primers. After using BsmI restriction enzyme, there were three patterns of genotypes representing the following DNA size bands: wild genotype which gave one band with a size of 822-bp; heterozygous genotype which gave three bands with sizes of 822-bp, 650-bp and 172-bp; and a homozygous genotype which gave two bands with sizes of 650-bp and 172-bp. For the TaqI restriction enzyme, there were three patterns of genotypes representing the following DNA size bands, wild genotype which gave one band with a size of 454-bp; a heterozygous genotype which gave three bands with sizes of 454-bp, 293-bp and 161-bp; and homozygous genotype which gave two bands with sizes of 293-bp and 161-bp. For the ApaI restriction enzyme, there were three patterns of genotypes representing the following DNA band sizes, wild genotype which gave one band with a size of 454-bp; heterozygous genotype which gave three bands with sizes of 454-bp, 237-bp and 217-bp; and a homozygous genotype which gave two bands with sizes of 237-bp and 217-bp.

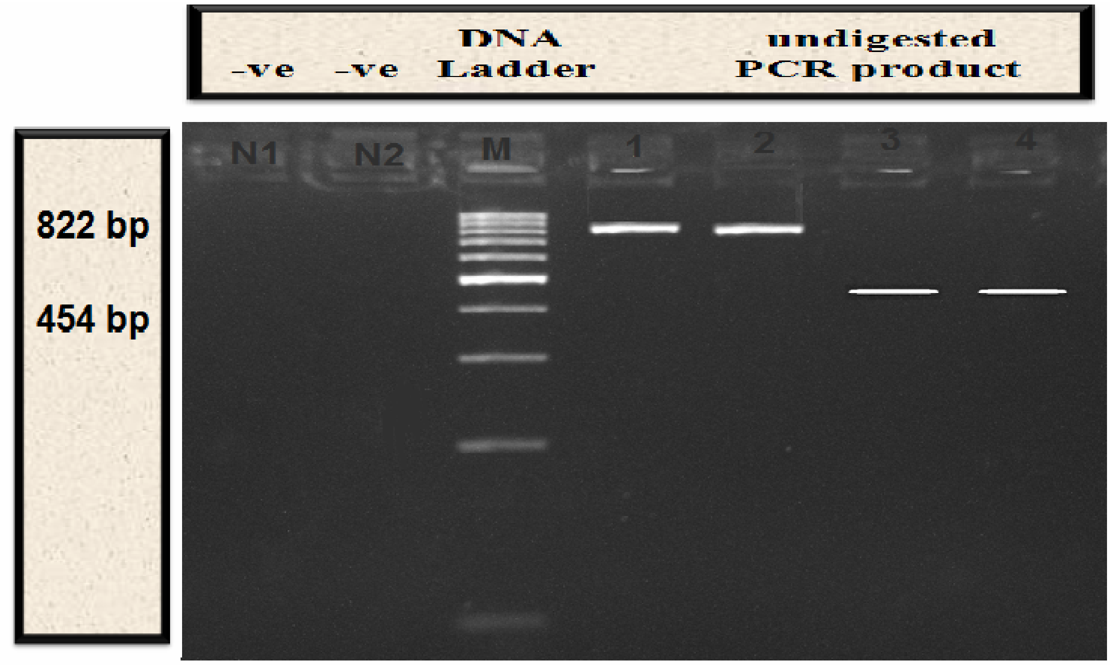


Figure 9: 2% Agarose gel electrophoresis of undigested PCR product for 4 samples. Lane M: 100-pb DNA ladder. Lanes N1 and N2: negative controls. Lanes 1 and 2 (822): BsmI PCR product. Lanes: 3 and 4: TaqI PCR product (454).

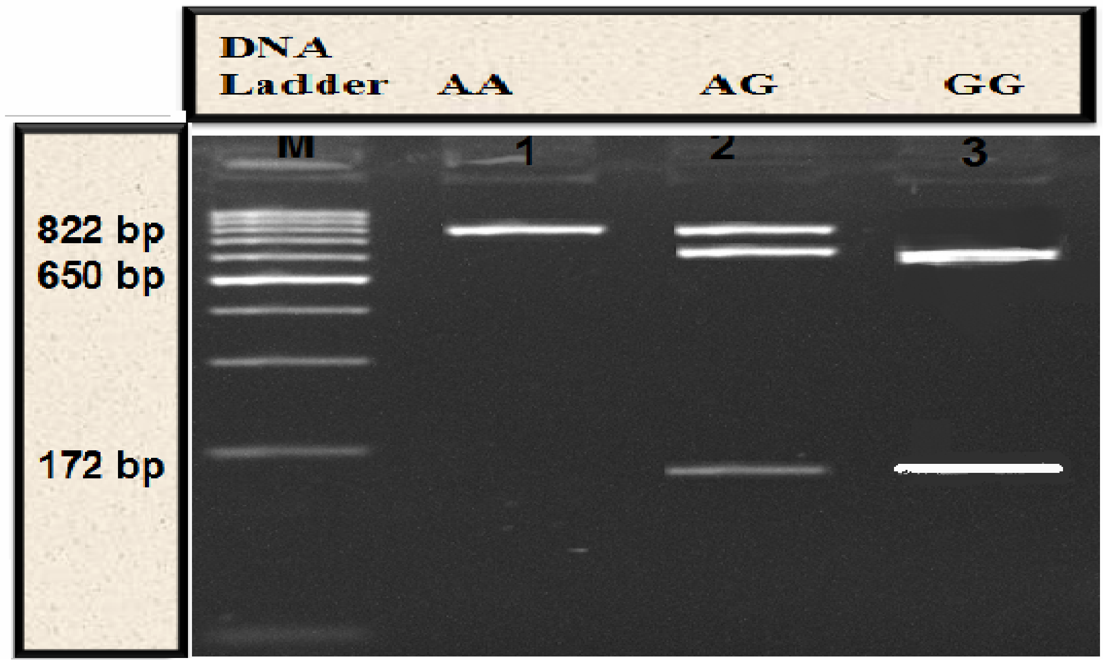


Figure 10: 2.5% Agarose gel electrophoresis of the BsmI RFLP products. Lane M: 100-pb DNA ladder. Lane 1: Homozygous wild type (AA) (822-bp). Lane 2: Heterozygous variant type (AG) (822, 650, 172-bp). Lane 3: Homozygous variant type (GG) (650, 172-bp).

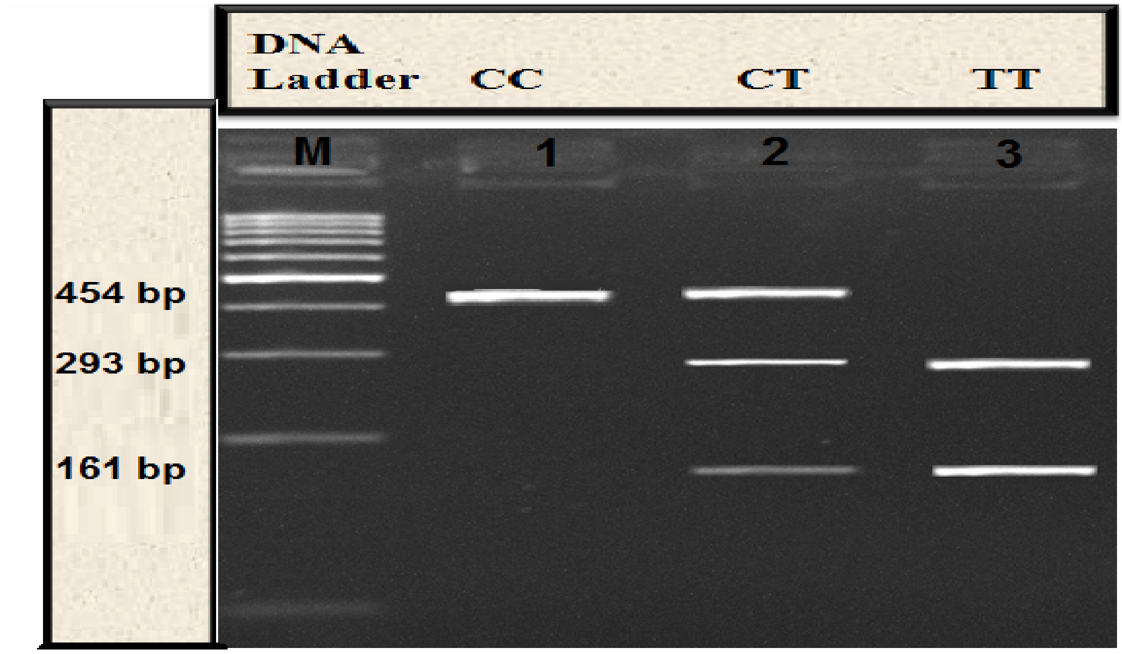


Figure 11: 3% Agarose gel electrophoresis of the TaqI RFLP products. Lane M: 100-pb DNA ladder. Lane 1: Homozygous wild type (CC) (454-bp). Lane 2: Heterozygous variant type (CT) (454, 293, 161-bp). Lane 3: Homozygous variant type (TT) (293, 161-bp).

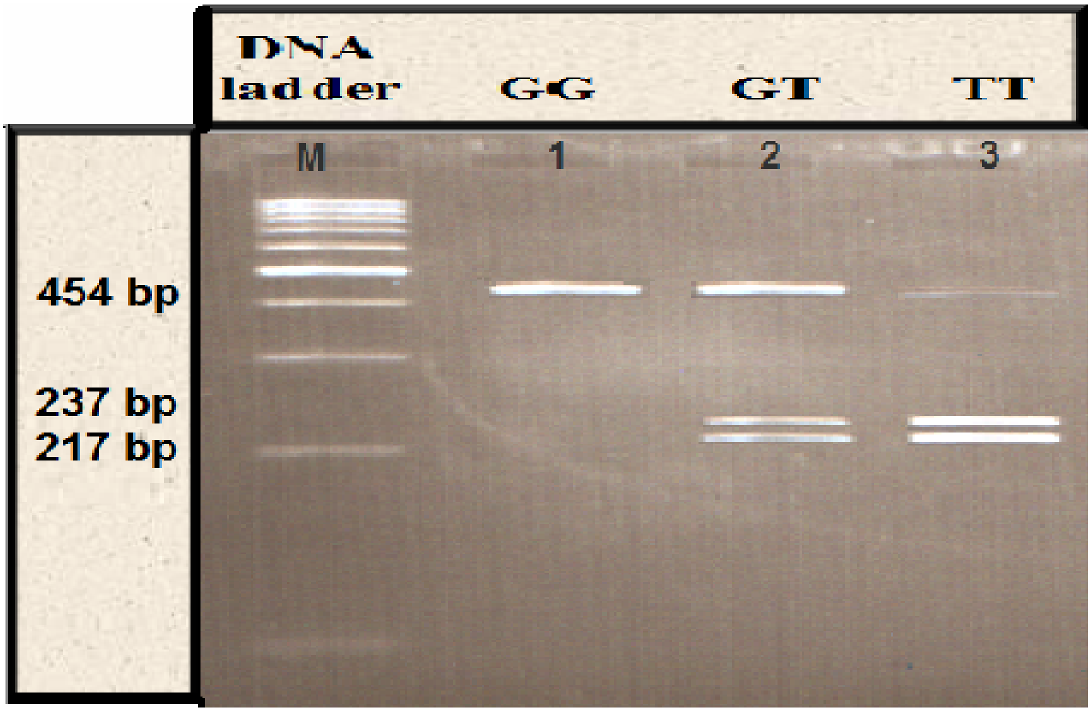


Figure 12: 3% Agarose gel electrophoresis of the ApaI RFLP products. Lane M: 100-pb DNA ladder. Lane 1: Homozygous wild type (GG) (454-bp). Lane 2: Heterozygous variant type (GT) (454, 237, 217-bp). Lane 3: Homozygous variant type (TT) (237, 217-bp).

3.2. Distribution of BsmI Allelic variants

The frequency of BsmI polymorphism in (200) diabetic patients and (200) controls is shown in Table 19. Seventy eight (39%) of the diabetic patients and fifty eight (29%) of the controls had a homozygous wild type (AA) for BsmI polymorphism, fifty eight (29%) of the diabetic patients and seventy nine (39.5%) of the controls had a heterozygous variant type (AG) of BsmI polymorphism, and sixty four (32%) of the diabetic patients and sixty three (31.5%) of the controls had a homozygous variant type (GG) of this polymorphism. The heterozygous variant genotype (AG) of BsmI polymorphism was significantly more frequent in controls than in patients (*p*-value =0.013; OR= 0.546; 95% CI= 0.338-0.882). The homozygous wild genotype (AA) of BsmI polymorphism was significantly more frequent in patients than in controls (*p*-value = 0.047, OR = 1, reference). No significant difference was observed between patients and controls in terms of the frequency of homozygous variant type (GG) (Table 19).

Table 19: The Association between BsmI genotypes polymorphism and diabetes

Genotype	Patients	controls	OR (95% CI)	p-value
AA	78 (39%)	58 (29%)	1 reference	0.047
AG	58 (29%)	79 (39.5%)	0.546 (0.338-0.882)	0.013
GG	64 (32%)	63 (31.5%)	0.755 (0.464-1.228)	0.258

Table 20: Allele frequency of BsmI polymorphism

Allele	Patients	Controls
A allele	214 (53.5 %)	195 (48.75 %)
G allele	186 (46.5 %)	205 (51.25%)

3.3. Distribution of TaqI Allelic Variants

The frequency of TaqI polymorphism in (200) diabetic patients and 200 controls is shown in Table 21. Seventy four (37%) of the diabetic patients and seventy three (36.5%) of the controls had a homozygous wild type (CC) for TaqI polymorphism, ninety six (48%) of the diabetic patients and eighty eight (44%) of the controls had a heterozygous variant type (CT) of TaqI polymorphism, and thirty (15%) of the diabetic patients and thirty nine (19.5%) of the controls had a homozygous variant type (TT) of this polymorphism. The difference between patients and controls in terms of the frequency of homozygous wild (CC) genotype, heterozygous (CT) variant genotype and homozygous (TT) variant genotype of TaqI polymorphism was not statistically significant (Table 21).

Table 21: The frequency of TaqI genotypes polymorphism

Genotype	Patients	Controls	OR (95% CI)	p-value
CC	74 (32%)	73 (36.5%)	1 reference	0.467
CT	96 (48%)	88 (44%)	1.076 (0.697-1.661)	0.740
TT	30 (15%)	39 (19.5%)	0.759 (0.427-1.349)	0.347

Table 22: Allele frequency of TaqI polymorphism

Allele	Patients	Controls
C Allele	244 (61%)	234 (58.5%)
T Allele	156 (39%)	166 (41.5%)

3.4. Distribution of ApaI Allelic Variants

Among 200 diabetic patients 90 (45%) were homozygous for the wild type (GG) of ApaI polymorphism, 83 (41.5%) were heterozygous for the variant type (GT) and 27 (13.5%) were homozygous for the variant type (TT) of ApaI polymorphism. Among 200 controls, 80 (40%) were homozygous for the wild type (GG) of ApaI polymorphism, 91 (45.5%) were heterozygous for the variant type (GT) and 29 (14.5%) were homozygous for the variant type (TT) of ApaI polymorphism (Table 23). Genotype frequencies of ApaI polymorphism did not significantly differ between patients and controls (Table 23).

Table 23: Association between ApaI genotypes polymorphism and diabetes

Genotype	Patients	Controls	OR (95% CI)	p-value
GG	90 (45%)	80 (40%)	1 reference	0.599
GT	83 (41.5%)	91 (45.5%)	0.811 (0.531-1.238)	0.331
TT	27 (13.5%)	29 (14.5%)	0.828 (0.452-1.515)	0.540

Table 24: Allele frequency of ApaI polymorphism

Allele	Patients	Controls
G Allele	263 (65.75%)	251 (62.75%)
T Allele	137 (34.25%)	149 (37.25%)

3.5. Further analysis

No significant difference was observed between male patients and male controls in terms of the BsmI, TaqI and ApaI genotypes polymorphisms (Table 25). No significant difference was observed between female patients and female controls in terms of the BsmI, TaqI and ApaI genotypes polymorphisms (Table 26)

Table 25: The frequency of BsmI, TaqI and ApaI genotypes among males

Genotype	Male (Patients) (n=96)	Male (Controls) (n=88)	*p*-value, OR (95%CI)
BsmI			
AA	43 (44.8)	29 (33.0%)	0.202 1, reference
AG	26 (27.1%)	33 (37.5%)	0.075, 0.531 (0.265-1.067)
GG	27 (28.1%)	26 (29.5%)	0.329, 0.700 (0.342-1.432)
TaqI			
CC	30 (31.2%)	27 (30.7%)	0.452, 1 (reference)
CT	50 (52.1%)	40 (45.5%)	0.729, 1.125 (0.578-2.19)
CC	16 (16.7%)	21 (23.9%)	0.374, 0.66 (0.298-1.577)
ApaI			
GG	46 (47.9%)	39 (44.3%)	0.850, 1 (reference)
GT	39 (40.6%)	37 (42.0%)	0.722, 0.894 (0.481-1.661)
TT	11 (11.5%)	12 (13.6%)	0.592, 0.777 (0.309-1.955)

Table 26: The frequency of BsmI, TaqI and ApaI genotypes among females

Genotype	Female (Patients) (n=104)	Female (Controls) (n=112)	*p*-value, OR (95%CI)
BsmI			
AA	35 (33.7%)	29 (25.9%)	0.251, 1 (reference)
AG	32 (30.8%)	46 (41.1%)	0.106, 0.576 (0.296-1.124)
GG	37 (35.6%)	37 (33.0%)	0.583, 0.829 (0.424-1.620)
TaqI			
CC	44 (42.3%)	46 (41.1%)	0.865, 1 (reference)
CT	46 (44.2%)	48 (42.9%)	0.995, 1.002 (0.562-1.786)
TT	14 (13.5%)	18 (16.1%)	0.617, 0.813 (0.361-1.831)
ApaI			
GG	44 (42.3%)	41 (36.6%)	0.650, 1 (reference)
GT	44 (42.3%)	54 (48.2%)	0.354, 0.759 (0.424-1.360)
TT	16 (15.4%)	17 (15.2%)	0.749, 0.877 (0.392-1.960)

No significant difference was observed between BsmI, TaqI and ApaI genotypes in terms of systolic blood pressure (Tables 27, 28, 29). A significant difference was, however, observed between BsmI genotypes in the terms of diastolic blood pressure; diastolic blood pressure was significantly higher in patients with heterozygous and homozygous variant genotypes (AG+GG) than in patients with homozygous wild genotype (AA) of this polymorphism (*p*=0.012, OR=1.032, 95%CI= (1.007-1.058)). Table (27)

Table 27: Association between BsmI genotype polymorphism and the systolic and diastolic blood pressure of diabetic patients

Genotype	Systolic blood pressure mean±SD	Diastolic blood pressure mean±SD
AA	129±22	73±13
AG+GG	132±20	78±11
***p*-value** **OR (95%CI)**	0.293 1.007 (0.994-1.021)	0.012 1.032 (1.007-1.058)

No association was observed between TaqI polymorphism and diastolic blood pressure (Table 28).

Table 28: Association between TaqI genotype polymorphism and the systolic and diastolic blood pressure of diabetic patients

Genotype	Systolic blood pressure mean±SD	Diastolic blood pressure mean±SD
CC	128±19	76±12
CT+TT	132±23	76±12
***p*-value** **OR (95%CI)**	0.145 1.010 (0.946-1.025)	0.854 0.998 (0.974-1.022)

Higher diastolic blood pressure was observed in patients with the heterozygous and homozygous variant genotypes (GT+TT) than in the homozygous wild genotype (GG) of ApaI polymorphism (p= 0.045, OR= 1.025, 95%CI= (1.001-1.049)) as shown in Table 29.

Table 29: Association between ApaI genotype polymorphism and the systolic and diastolic blood pressure of diabetic patients

Genotype	Systolic blood pressure mean±SD	Diastolic blood pressure mean±SD
GG	128±23	74±12
GT+TT	132±20	77±12
***p*-value** **OR (95%CI)**	0.161 1.010 (0.996-1.023)	0.045 1.025 (1.001-1.049)

No significant association was observed between the BsmI, TaqI and ApaI genotype polymorphisms and FBS and no significant association was observed between the BsmI, TaqI and ApaI genotypes polymorphisms and HbA1c levels (Tables 30).

Table 30: Association between BsmI, TaqI and ApaI genotypes polymorphism and FBS and HbA1c tests of diabetic patients

Genotype	FBS mg/dl	HbA1c%
BsmI		
AA	171±72	7.8±1.7
AG+GG	166±73	7.7±1.5
p-value **OR (95%CI)**	0.622 0.999 (0.995-1.003)	0.719 0.967 (0.805-1.161)
TaqI		
CC	170±82	7.7±1.5
CT+TT	167±66	7.7±1.6
p-value **OR (95%CI)**	0.738 0.999 (0.495-1.003	0.741 1.032 (0.856-1.245)
ApaI		
GG	165±67	7.7±1.6
GT+TT	170±77	7.7±1.5
p-value **OR (95%CI)**	0.634 1.001 (0.997-1.005)	0.841 0.982 (0.820-1.176)

3.6. Vitamin D receptor polymorphism and disease variables and complications

No significant difference was observed between diabetic patients with hypertension and diabetic patients without hypertension in terms of BsmI, TaqI, ApaI, genotypes polymorphisms (Table 31).

Table 31: The frequency of BsmI, TaqI and ApaI genotypes polymorphism among hypertensive diabetic patients and normotensive diabetic patients

Genotype	Hypertensive diabetic patients (N=156)	Normotensive diabetic patients (N=44)	*p*-value
BsmI			
AA	65 (41.7%)	13 (29.5%)	0.303
AG	42 (26.9%)	16 (36.4%)	0.127
GG	49 (31.4%)	15 (34.1%)	0.315
TaqI			
CC	57 (36.5%)	17 (38.6%)	0.749
CT	74 (47.4%)	22 (50.0%)	0.993
TT	25 (16.0%)	5 (11.4%)	0.477
ApaI			
GG	74 (47.4%)	16 (36.4%)	0.057
GT	58 (37.2%)	25 (56.8%)	0.059
TT	24 (15.4%)	3 (6.8%)	0.415

A statistically significant difference was observed between obese diabetic patients and nonobese diabetic patients in terms of BsmI polymorphism; nonobese diabetic patients had higher (AA) wild genotype of BsmI polymorphism than obese diabetic patients

(*p*= 0.049, OR= 1, (reference)) and obese or overweight diabetic patients had higher (GG) homozygous variant genotype of the polymorphism than non obese patients (*p*= 0.019, OR= 3.540, (95%CI= 1.234-10.156)). No significant difference was observed between obese or overweight diabetic patients and non obese diabetic patients in terms of TaqI and ApaI genotypes polymorphisms, except for the observation that obese or overweight diabetic patients had higher (CT) heterozygous variant genotype of TaqI polymorphism than nonobese patients (*p*-value= 0.019, OR = 0.315, (95% CI = 0.120-0.827)) (Table 32).

Table 32: The frequency of BsmI, TaqI and ApaI genotypes polymorphism among obese or overweight diabetic patients and nonobese diabetic patients

Genotype	Obese or overweight-diabetic patients (N=169)	Non- obese- Diabetic patients (N=31)	*p*-value OR (95%CI)
BsmI			
AA	60 (35.5%)	18 (58.1%)	0.049 1, reference
AG	50 (29.6%)	8 (25.8%)	0.177 1.875 (0.752-4.674)
GG	59 (34.9%)	5 (16.1%)	0.019 3.540 (1.234-10.156)
TaqI			
CC	68 (40.2%)	6 (19.4%)	0.055 1, reference
CT	75 (44.4%)	21 (67.7%)	0.019 0.315 (0.120-0.827
TT	26 (15.4%)	4 (12.9%)	0.417 0.574 (0.150-2.98)
ApaI			
GG	72 (42.6%)	18 (58.1%)	0.163 1, reference
GT	71 (42%)	12 (38.7%)	0.338 1.479 (0.664-3.294)
TT	26 (15.4%)	1 (3.2%)	0.075 6.500 (0.826-51.152)

No significant difference was observed between diabetic patients that have coronary artery disease (CAD) and diabetic patients without coronary artery disease in terms of BsmI, TaqI, ApaI, genotypes polymorphisms (Table 33).

Table 33: The frequency of BsmI, TaqI and ApaI genotypes polymorphism among diabetic patients with and without coronary artery disease.

Genotype	CAD-diabetic patients (n=33)	diabetic patients without CAD (n=167)	*p*-value OR (95%CI)
BsmI			
AA	12 (36.4%)	66 (39.5%)	0.333
AG	7 (21.2%)	51 (30.5%)	0.582
GG	14 (42.4%)	50 (29.95%)	0.322
TaqI			
CC	16 (48.5%)	58 (34.7%)	0.284
CT	14 (42.4%)	82 (49.1%)	0.235
TT	3 (9.1%)	27 (16.2%)	0.175
ApaI			
GG	12 (36.4%)	78 (46.7%)	0.311
GT	14 (42.4%)	69 (41.3%)	0.517
TT	7 (21.2%)	20 (12.0%)	0.126

DISSCUSION

Vitamin D is a group of fat soluble secosteroid that function in humans as a prohormone (Zhang *et al*., 2010). Vitamin D regulates calcium and phosphate metabolism and promotes healthy growth and remodeling of bone (Heaney *et al.,* 2003). Sufficient concentrations of serum vitamin D prevent rickets in children and osteomalacia in adults (Kumar *et al*., 2009, Holick, 2006). Moreover, vitamin D seems to be involved and is required for normal functioning of almost all organ systems of the human body (Nejentensev *et al*., 2004). It also affects neuromuscular function and it influences the functions of genes that regulate proliferation, differentiation and apoptosis of cells (Holick, 2007).

Vitamin D is converted in the kidney or by macrophages into calcitriol, the biologically active form that binds to vitamin D-binding protein (Holick, 2004). Vitamin D exerts its effects via a receptor, vitamin D receptor (VDR), which is located in the nucleus of the target cells (Muszkat *et a*l., 2010). Binding of calcitriol to VDR allows it to act as a transcriptional factor that modulates gene expression of proteins that are regulated by vitamin D (Muszkat *et al*., 2010). Since vitamin D exerts its effects through the vitamin D receptor, nucleotide changes in the VDR gene may affect the functional integrity of the VDR protein. In such way that downstream vitamin D pathways are adversely affected (Utterlinden *et al.,* 2004). Most of these nucleotide changes in the VDR gene occur as single nucleotide polymorphisms which have been associated with diseases like diabetes, cancer and cardiovascular disease (Utterlinden *et al.,* 2004). This study tested the hypothesis that VDR gene polymorphisms have a role in the pathogenesis of type 2 diabetes mellitus. Three VDR polymorphisms (BsmI, ApaI and TaqI) have been examined in this study.

1. Vitamin D Deficiency

Vitamin D deficiency is a serious global problem that is assuming epidemic proportions (Lips, 2001). Studies from the United States, Europe, India, Australia, South America, and Southeast Asia have shown that approximately 50 percent of children and adults are at high risk of vitamin D deficiency (Zittermann *et al*., 2006). Concerning vitamin D status, alarming results have recently been reported in urban dwellers in Europe. In a Hungarian study, middle-aged urban dwellers had only modest seasonal variations in circulating 25(OH)D levels and a high percentage of subjects had a low vitamin D status throughout the year, despite marked seasonal fluctuations in daily sunshine**.** The prevalence of 25(OH)D levels below 20 ng/ml during spring, summer, autumn, and winter was 71.0%, 46.3%, 49.4%, and 56.7%, respectively (Bhattoa *et al.,* 2004). Similarly, white British people referring to an outpatient clinic at a city hospital had mean 25(OH)D levels around 20 ng/ml throughout the year without seasonal fluctuations (Pal *et al*., 2003). A high prevalence of insufficient or deficient 25(OH)D levels is also reported in dark-skinned people living in Northern latitudes. Mean 25(OH)D levels below 20 ng/ml have been observed in African Americans (Nesby-O'Dell *et al.,* 2002). Moreover, mean 25(OH)D levels below 12.5 ng/ml in summer and below 6.25 ng/ml in winter have been reported in South Asian immigrants in Great Britain (Pal *et a*l., 2003). In Lebanon, 25(OH)D levels below 9.7 ng/ml were observed in 72.8% of a middle-aged group of Adults (Gannage-Yared *et al*., 2000). Very low mean 25(OH)D levels of 4 ng/mll in winter and 8 ng/ml in summer were observed in Indian physicians and nurses who lived in the city of Delhi and had a daily sun exposure of only 25 min. In contrast, Indian soldiers with a daily sun exposure of 370 min had mean 25(OH)D level of 20 ng/ml in winter (Goswami *et al.,* 2000). Vitamin D deficiency is a common finding

that has been linked to type 2 diabetes mellitus in humans many years ago (chiu *et al.*, 2004), and many studies have suggested that low 25(OH)D levels are associated with hypertension, diabetes mellitus, insulin resistance and an elevated BMI. (Melamed *et al.*, 2008).

In this study, vitamin D levels were significantly lower in controls than in patients. It was a surprising finding that male and female diabetic patients had a higher vitamin D levels than male and female controls. Vitamin D levels were significantly higher in patients than in controls because some of these patients received vitamin D supplements. Although vitamin D levels were higher in diabetic patients, they did not contribute to a lower the risk of diabetes. Results of this study are in contrast to other studies that found that vitamin D levels were significantly decreased in patients with type 2 diabetes mellitus than in controls (Scragg *et al.*, 1995, Isaia *et al.*, 2001, Yoho *et al.*, 2009). Despite the fact that Jordan is a sunny country most of the year and sufficient exposure to sunlight is guaranteed for those who seek it, diabetic patients and controls enrolled in this study had low vitamin D levels.

No association was observed between vitamin D levels and fasting blood sugar or between vitamin D levels and HbA1c values. These observations are in contrast to a study carried out in Japan that found that serum 25 (OH)D3 in Japanese type 2 diabetic patients correlated inversely with HbA1c levels (Suzuki *et al.*, 2006). Other studies have also found that plasma 25 (OH)D3 concentrations were inversely associated with fasting glucose levels (Lui *et al.*, 2009, Honeyman *et al.*, 2007, Batieha *et al.,* 2011).

Hypertension is a common co-morbidity among diabetic patients (Mubarak *et al.,* 2008) and has been linked to vitamin D deficiency (Schmitz *et al.*, 2009). However, no significant difference was observed between hypertensive diabetic patients and normotensive diabetic patients in this study with respect to vitamin D levels. One

study have reported similar observation (Forouhi *et al*., 2008, Forman *et al*., 2005), but it is in contrast to other studies that have found that serum 25 hydroxyvitamin D_3 and blood pressure correlated inversely (Scragg *et al*., 2007, Schmitz *et al*., 2009, Anderson *et al*., 2010) and that short-term supplementation with vitamin D and calcium reduced systolic blood pressure (Pfeifer *et al*., 2001).

Several experimental studies have demonstrated that the vitamin D hormone calcitriol plays a pivotal role in the normal functioning of cardiomyocytes and the vasculature (Michos and Melamed, 2008). Low vitamin D metabolite levels are associated with various coronary artery disease outcome such as myocardial infarction, congestive heart failure, and cardiovascular mortality (Zittermann *et al*., 2008, Michos and Melamed, 2008). In this study, no significant difference was found between diabetic patients with coronary artery disease and diabetic patients without coronary artery disease in term of vitamin D levels. This could be due to the small number of diabetic patients with coronary artery disease as compared to diabetic patients without coronary artery disease. Rajasree *et al*. (2001) found that serum levels of 25 (OH)D3 is elevated in a higher proportion of patients with either angiographic evidence of coronary artery disease or patients with acute myocardial infarction, compared to controls. To the contrary, other studies have reported that low 25 (OH)D3 levels are associated with coronary artery disease (Scragg *et al*., 1990, Melamed *et al*., 2008). Vitamin D is believed to be important in maintaining cardiovascular health. (Bonakdaran and Varasteh, 2009) and vitamin D levels were highly associated with coronary artery disease, myocardial infarction, heart failure, and stroke (Anderson *et al*., 2010, Poole *et al.,* 2006).

No significant difference was observed between obese diabetic patients and non obese diabetic patients in this study in terms of vitamin D levels which is in contrast to what

reported by Arunabh *et al.,* (2003) who concluded that the percentage body fat content is inversely related to serum vitamin D levels. Results of this study could not be confirmed because there was a big difference between the number of obese diabetic patients and non obese diabetic patients.

5. Frequency of BsmI, TaqI and ApaI polymorphisms among the population of this study

An ethnic variation in the distribution of BsmI, TaqI and ApaI polymorphisms was observed. According to Uitterlinden *et al*. (2004), the (A) wild type allele of BsmI polymorphism being much higher in the Caucasians (42%) than in the Asians (7%) and high among African Americans (36%). The (C) wild type allele of TaqI polymorphism is much higher in the Caucasians (43%) than in the Asians (8%) populations but is intermediate among African Americans (31%). The (G) wild type allele of ApaI polymorphism was, according to the previous study, much higher in Asians (74%) than in African Americans (31%), but is intermediate among Caucasians (44%). In the present study, the frequency of the wild type alleles of the three polymorphisms among patients and controls was different where BsmI-(A) frequency was 53.5% as compared to 48.75%, TaqI-(C) frequency was 61% as compared to 58.5%, ApaI-(G) frequency being 65.75% as compared to 62.75%.

2.1. BsmI, TaqI and ApaI polymorphisms and its association with diabetes mellitus

The presence of vitamin D receptors (VDR) in pancreatic tissue and the relationship between certain allelic variations in the VDR gene with glucose tolerance and insulin secretion have supported the hypothesis that there is a role for vitamin D receptor polymorphism in the pathogenesis of type 2 diabetes mellitus (Palomer *et al*., 2008).

This study tested the hypothesis that VDR gene polymorphism has a role in pathogenesis of type 2 diabetes mellitus. Polymorphisms of vitamin D receptor (VDR) gene were analyzed by DNA amplification with polymerase chain reaction (PCR) and endonuclease digestion with BsmI, TaqI, and ApaI enzymes since these techniques are suitable for this type of a study.

No statistically significant difference was found between the frequency of BsmI, TaqI and ApaI polymorphisms among diabetics and nondiabetics, which is in agreement with reports made by others (Dilmec *et al*., 2010, Maleki *et al*., 2003, Ye *et al.,* 2001, Boullu-Sanchis *et al.,* 1999, Barrett-Connor, 2002, Bid *et al*., 2009). The frequency of (AG) heterozygous variant genotype of BsmI polymorphism was, however, significantly higher in controls than in patients (p=0.013, OR= 0.546, 95%CI= (0.338-0.882)); which suggests that the heterozygous variant type of BsmI polymorphism is associated with reduced risk of type 2 diabetes mellitus, a finding that to our knowledge has never been reported before. Other studies have shown an association between the ApaI polymorphism (homozygosis for the G wild type allele) and lower insulin secretion in a healthy Bangladeshi Asian population (Hitman *et al*., 1998). Ogunkolade *et al*. (2002) have also shown a positive association between the TaqI (CC homozygous wild type) and the BsmI (GG homozygous variant type) polymorphisms with reduced insulin secretory capacity in the Bangladeshi Asian population. Speer *et al*. (2001) reported that patients with diabetes with the (AA) wild genotype of the

BsmI polymorphism of the VDR gene presented with higher levels of postprandial serum C-peptide, which points to a possible role in the pathogenesis of type 2 diabetes. No association was found in this study between the three polymorphisms and fasting plasma glucose or HbA1c, which is in contrast to other studies that have found a correlation between ApaI polymorphism and fasting plasma glucose and glucose intolerance in a community-based study of older adults (Oh and Barrertt-Connor 2002). More recently, it has been reported that VDR (A) wild type allele of the BsmI polymorphism is associated with elevated fasting glucose in healthy young men long before the onset of type 2 diabetes (Ortlepp *et al*., 2003).

2.2. Association between hypertension and BsmI, TaqI and ApaI genotypes polymorphisms in diabetic patients.

No significant difference was observed between hypertensive diabetic patients and normotensive diabetic patients included in this study in terms of BsmI, TaqI, and ApaI polymorphisms. Diastolic blood pressure was, however, higher in diabetic patients with (AG+GG) heterozygous and homozygous variant genotypes than in diabetic patients with the (AA) homozygous wild genotype of the BsmI polymorphism. Diastolic blood pressure was also higher in diabetic patients with (GT+GG) heterozygous and homozygous variant genotype than in the (GG) homozygous wild genotype of the ApaI polymorphism. No difference was observed with respect to the TaqI genotypes. Muray *et al.* (2003) found that systolic blood pressure was higher in men with the (GG) homozygous variant genotype of the BsmI polymorphism than in the other genotypes. However, it is important to point out here that the reliability of these results is questionable because of the small number of normotensive diabetic patients as compared to hypertensive diabetic patients.

2.3. Association between obesity and BsmI, TaqI and ApaI genotypes polymorphisms in the diabetic patients.

A statistically significant difference was found between obese diabetic patients and nonobese diabetic patients included in this study in terms of BsmI polymorphism; nonobese diabetic patients had higher prevalence of (AA) wild genotype of BsmI polymrphism than obese diabetic patients and obese or overweight diabetic patients had higher prevalence of (GG) homozygous variant genotype of the polymorphism than nonobese patients. No significant difference was observed between obese or overweight diabetic patients and nonobese diabetic patients of this study in terms of TaqI and ApaI genotypes polymorphisms, except for the observation that obese or overweight diabetic patients had higher prevalence of (CT) heterozygous variant genotype of TaqI polymrphism than nonobese patients. Ye *et al.,* (2001) provided evidence that polymorphisms in the VDR gene might be associated with susceptibility to obesity in subjects with early-onset T2DM. He also observed that the presence of the (CC) homozygous wild genotype of TaqI polymorphism or of the (GG) homozygous variant genotype of the BsmI polymorphism accounted for a 30% increase in the prevalence of obesity in T2DM subjects, whereas Filus *et al*. (2008), found that (AA) homozygous wild type of BsmI polymorphism carriers tend to have higher body mass index (BMI) compared with the (GG) homozygous variant genotypes of BsmI polymorphism. In contrast, Tworowska-Bardzińska *et al.* (2008) reported that the BsmI polymorphism in the VDR gene does not seem to predispose to obesity.

2.4. Association between coronary artery disease and BsmI, TaqI and ApaI genotypes polymorphisms in the diabetic patients.

No statistically significant difference was observed between the prevalence of BsmI, TaqI and ApaI genotypes and the presence of coronary artery disease in the diabetic

patients included in this study. No significant difference was observed with respect to these polymorphisms between diabetic patients with CAD and those without CAD. These results are in agreement with results reported by other studies (Pan *et al*., 2009, Shanker *et a*l., 2011), but they are in contrast to another study that found that patients with the (AA) homozygous wild genotype of the BsmI polymorphism had higher risk of developing CAD (Ortlepp *et al*., 2001). Since the proportion of patients with coronary artery disease was very low among the diabetic patients of this study, it was not possible to properly assess the impact of these polymorphisms on this disease between diabetic patients.

CONCLUSIONS

On the basis of the results of this study, the following conclusions can be made:

1. Vitamin D deficiency seems to be common even among apparently healthy individuals.
2. The heterozygous variant genotype (AG) of BsmI polymorphism seems to reduce the risk of type 2 diabetes mellitus, whereas the other vitamin D receptor gene polymorphisms seem to have no effect in the pathogenesis of type 2 diabetes mellitus.
3. Vitamin D receptor polymorphisms seem to have no effect in the pathogenesis of coronary artery disease among diabetic patients.
4. Vitamin D receptor polymorphisms might play a role in the pathogenesis of hypertension and obesity among diabetic patients.
5. The free wild alleles are higher in our population comparison to the other populations.

RECOMMENDATIONS

On the basis of the conclusions made, the following recommendations can be made:

1. More polymorphisms of VDR gene known to predispose to diabetes need to be studied.
2. Carry out research that involves the increase of sample size by recruiting more Jordanian diabetic patients and age matched controls to confirm or refute our findings.
3. Use more proper laboratory measurements to quantify insulin resistance and β cell function (HOMA test).

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APPENDECIES

Appendix A

Written Informed consent

موافقة على سحب عينة دم لغايات البحث العلمي

اسم الباحث : داليا خليل محمد الحنش.

مكان إجراء البحث : الجامعة الأردنية- كلية الطب- قسم علم الأمراض و الأحياء الدقيقة والطب الشرعي.

أخي المتبرع : أرجو التكرم بالاطلاع على ما يلي :

هذه الدراسة هي إحدى الدراسات الطبية التي تقوم بها كلية الطب في الجامعة الأردنية، وسوف يتم أخذ عينة دم لغاية تحليلها في مختبرات كلية الطب لمعرفة التنوع الشكلي لمورثة مستقبل فيتامين (د) لدى مرضى أردنيين مصابين بداء السكري من النوع الثاني.

لا توجد خطورة على المتبرع سوى أن عملية سحب الدم قد تحمل المخاطر الإعتيادية لسحب الدم و هي نادرة الحدوث.

في حال الموافقة على إعطاء عينة دم، سيبقى اسمك طي الكتمان وسيتم التعامل مع عينة الدم بطريقة الترميز.

و تجدر الإشارة أن لجنة الأخلاق الطبية في الجامعة الأردنية قد وافقت على هذا البحث.

ملاحظة : يحق لك الإنسحاب من الدراسة متى شئت، و سوف يتم التعامل مع العينة عن طريق الترميز من دون ذكر اسم المتطوع.

موافقة المتطوع: أنا المتطوع ______________________________ قد قرأت المعلومات المذكورة

أعلاه و فهمتها، وبناء عليه فإني حرا مختارا، أوافق على إعطاء عينة دم للبحث العلمي بعنوان :

التنوع الشكلي لمورثة مستقبل فيتامين (د) لدى مرضى أردنيين مصابين بمرض السكري من النوع الثاني

وعلى ذلك أوقع

التوقيع:

Appendix B

Data Collection Form for Patients

Date:

Patient from Type 2 diabetes mellitus

Name: Serial No.:

Nationality: Patients hospital No.:

Gender: M F Age:

Weight: (Kg) Height: (m) BMI:

Blood pressure: (mmHg)

Fasting Blood Sugar (FBS): (mg/dl) HbA1C: %

Vitamin D levels: (ng/ml)

Ca: (mg/dl)

Urea: (mg/dl)

Creatinine: (mg/dl)

Ca: (mg/dl)

Na: (mmol/L)

K: (mmol/L)

Other diseases: ____

Appendix C

Preparation of 10X Tris-boric acid EDTA

Preparation of 10X Tris-boric acid EDTA (TBE) buffer, pH 8.2. To 1 litter distilled water the following were added:

- 108 g Tris-base.
- 55 g Boric acid.
- 7.3 g EDTA.

These ingredients were then completely dissolved with magnetic stirrer until the solution became clear. 1X-TBE buffer was prepared by dilution of 10X TBE buffer.

التنوع الشكلي لمورثة مستقبل فيتامين (د) لدى مرضى أردنيين مصابين بداء السكري من النوع الثاني

إعداد
داليا خليل الحنش

المشرف
الأستاذ الدكتور عزمي محافظة

ملخص

لقد تبين أن نقص فيتامين (د) يلعب دورا مهما في الإصابة بعدة أمراض. لقد كان الهدف من هذه الدراسة هو تحديد تكرار التنوع الشكلي لمورثة مستقبل فيتامين (د) لدى مرضى أردنيين مصابين بداء السكري من النوع الثاني و استقصاء العلاقة بين هذا التنوع الشكلي ومتغيرات المرض.
اشتملت الدراسة على مئتين من المرضى المصابون بداء السكري من النوع الثاني الذين يراجعون عيادات السكري في مستشفى الجامعة الأردنية والمركز الوطني للسكري والغدد الصم والوراثة، و مئتين من الأشخاص غير المصابين بداء السكري ولا يعانون من السمنة والذين خدموا كعينة ضابطة. ولقد تم الحصول على موافقة كتابية لكل مشارك في هذه الدراسة. تم جمع عينات الدم واستخلاص الحمض النووي الجيني. وقد تم تحليل الأشكال المتعددة لمورثة مستقبل الفيتامين (د) (VDR) بواسطة تفاعل البلمرة المتسلسل (PCR) والهضم بأنزيمات النيوكلازا الداخلية BsmI وTaqI و ApaI. تم الحصول على البيانات السريرية والمخبرية بما في ذلك الوزن و الطول و ضغط الدم الانقباضي والانبساطي والسن والأمراض الأخرى التي عند المرضى و نسبة السكر في الدم عند الصيام و نسبة السكر التراكمي HbA1c واليوريا والكرياتينين، والصوديوم والبوتاسيوم والكالسيوم وفيتامين (د) من الملفات الطبية للمرضى. كانت نسبة فيتامين (د) أعلى

عند مرضى السكري منها عند المجموعة الضابطة و كانت نسبة فيتامين (د) أعلى عند الذكور و الإناث المرضى منها عند الذكور و الإناث من المجموعة الضابطة في حين لم تكن هنـــاك أي علاقة بين نسبة فيتامين (د) و نسبة السكر في الدم عند الصيام او نسبة السكر التراكمي لم تكـــن هناك أي علاقة بين نسبة فيتامين (د) و الإصابة بارتفاع التوتر الشرياني و الـــسمنة و أمـــراض الشريان التاجي. ولم يكن هناك أي فرق ذو دلالة إحصائية في نسبة توزيع الأنمـــاط الجينيـــة الثلاثة BsmI وTaqIو ApaI بين الأشخاص المصابين و غيـــر المـــصابين بالـــسكري (p≥ 0.05). و لكن كانت نسبة تكرار التباين الجيني Bb من BsmI أعلى لدى المجموعة الضابطة (p=0.013). لم يكن هناك أي فرق ذو دلالة إحصائية في نسبة توزيع الأنماط الجينية الثلاثة BsmI وTaqIو ApaI بين الذكور و الإناث المرضى و الذكور و الإنـــاث مـــن المجموعـــة الضابطة ولم يكن هناك اي فرق في معدل التوتر الشرياني الإنقباضي بالنسبة للأنمـــاط الجينيـــة الثلاثة و لكن كان هناك فرق في معدل الضغط الانبساطي بالنسبة للتنوعات الـــشكلية BsmI و ApaI حيث كان معدل الضغط الإنبساطي أعلى في الأنماط الجينية (GA+GG) و (GT+TT) للتنوعات الجينية BsmI و ApaI على التوالي. ولم يكن هناك أي علاقة بين الأنمـــاط الجينيـــة الثلاثة و نسبة السكر في الدم عند الصيام و نسبة السكر التراكمي. إضافة إلى ذلك لم يكن هناك أي فرق ذو دلالة إحصائية في نسبة توزيع الأنماط الجينية الثلاثة BsmI وTaqIو ApaI بين الأشخاص المصابين بارتفاع التوتر الشرياني وغير المصابين بارتفاع التوتر الـــشرياني و بـــين الأشخاص المصابين بأمراض الشريان التاجي وغير المصابين به من مرضـــى الـــسكري (p≥ 0.05). كانت هناك علاقة بين التنوعين الشكليين BsmI و TaqI و السمنة ,حيث كانت نـــسبة تكرار التباين الجيني (GG) من التنوع الشكلي BsmI والتباين الجيني (CT) من التنوع الشكلي TaqI أعلى عند مرضى السكري الذين يعانون من البدانة أو الزيادة في الوزن مـــن مرضـــى السكري الغيربدناء, و كانت نسبة تكرار التباين الجيني (AA) من التنوع الشكلي BsmI أعلـــى عند مرضى السكري غير البدناء.

بناءا على نتائج هذه الدراسة فإنه يمكن الإستخلاص بأنه لا يبدو أن هناك اي علاقة بين التنوع الشكلي لمورثة فيتامين (د) ومرض السكري و لكن يبدو انه يوجد علاقة بين هذا التنوع الشكلي و الإصابة بالسمنة وارتفاع التوتر الشرياني. أيضا يبدو أنه لاتوجد علاقة بين التنوع الشكلي لمورثة فيتامين (د) ومرض الشريان التاجي.